“Natural abilities are like natural plants, that need pruning by study”
by Bacon, Francis

Members of the jury

1. Prof. Yue Dai, Department of Pharmacognosy of Chinese Material Medica, School of Traditional Chinese Pharmacy, China Pharmaceutical University, China

2. Prof. Dr. ir. Norbert De Kimpe, Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Belgium (**promoter**)

   Prof. Zheng-tao Wang, Department of Pharmacognosy, School of Traditional Chinese Pharmacy, China Pharmaceutical University, China (**promoter**)

3. Prof. Dr. Stefaan De Smet, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Belgium

4. Prof. Dr. Tom Desmet, Centre for Industrial Biotechnology and Biocatalysis, Faculty of Bioscience Engineering, Ghent University, Belgium

5. Prof. Ling-yi Kong, Department of Natural Medicinal Chemistry, School of Traditional Chinese Pharmacy, China Pharmaceutical University, China

6. Prof. Jian-xin Li, Department of Organic Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, China

7. Prof. Wei-lin Li, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, China

8. Prof. Bo-yang Yu, Department of Pharmacognosy, School of Traditional Chinese Pharmacy, China Pharmaceutical University, China

Promoters: Prof. Dr. ir. Norbert De Kimpe
Department of Sustainable Organic Chemistry and Technology,
Faculty of Bioscience Engineering, Ghent University

Prof. Zheng-Tao Wang
Department of Pharmacognosy,
China Pharmaceutical University

Dean: Prof. Dr. ir. Guido Van Huylenbroeck

Rector: Prof. Dr. Paul Van Cauwenberge

MSc. Jian Chen

STUDY ON THE ISOLATION, CHARACTERIZATION AND POSSIBLE BIOACTIVITY OF CONSTITUENTS WITH HYPOGLYCEMIC ACTIVITY FROM *GYNURA DIVARICATA* AND *G. BICOLOR*

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Chemistry

Dutch translation of the title:

Studie van de isolatie, karakterisering en mogelijke bioactiviteit van bestanddelen met hypoglycemische activiteit van *Gynura divaricata* en *G. bicolor*.

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Ghent, April 2013

The author, The promoter, The promoter,

MSc. Jian Chen Prof. Dr. ir. Norbert De Kimpe Prof. Zheng-Tao Wang

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Preface

Upon the termination of my Ph.D. thesis, I would like to take this opportunity to express my sincere gratitude, from my heart, to all the individual persons who have contributed to the completion of this work.

First of all, I deeply appreciate my promoter, Prof. Dr. ir. Norbert De Kimpe for the opportunity he offered me to study in Ghent University and his supervision and critical review throughout the course of my doctoral research work. I still remember the first time we met in Nanjing in June 2005, and until now we have worked together for a long time. I am grateful to him for sharing with me the serious way to do the science, which I will keep in mind at my academic career in the future. I also would like to thank my promoter Prof. Zheng-Tao Wang, for his guidance and sharing knowledge on the isolation of natural products. Even I was abroad, he would discuss with me on the thesis for hours by phone. I am very thankful to him. Their supervision, criticism and encouragement were so important to me that I definitely could not complete this work without them.

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I would like to thank the members of the reading committee:

1. Prof. Dr. Tom Desmet, Centre for Industrial Biotechnology and Biocatalysis, Faculty of Bioscience Engineering, Ghent University, Belgium

2. Prof. Ling-yi Kong, Department of Natural Medicinal Chemistry, School of Traditional Chinese Pharmacy, China Pharmaceutical University, China

3. Prof. Jian-xin Li, Department of Organic Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, China

for their critical evaluation of my thesis.

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No words can describe my gratitude to my wife Windy. She sacrificed a lot during these years because I was always away from home. Her understanding, tolerance, love and endless encouragement always support me to continue the doctoral work. I dedicated this work to you because I couldn't have accomplished it without you.

Last but not the least, I would not forget to thank my dear parents. You are an inspiration for me to be a strong man, a real father. And I have learned from you what is responsibility. My little son Yilin, although not yet old enough to understand what I am writing right now, I want to say sorry to you because I owed you a complete childhood when I was abroad. I wish I can make complement to you in the rest of our life.

Should there be anyone I failed to acknowledge, my sincere regret, my apology and my thanks go to him / her too.

Jian
April, 2013

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Chapter 1

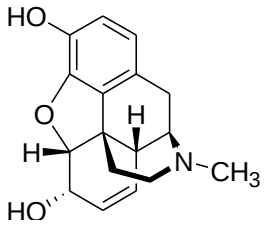
GENERAL INTRODUCTION

1.1 Natural products

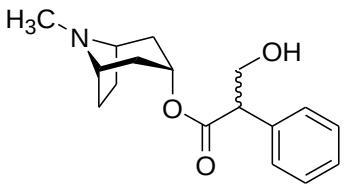
Chemical substances derived from plants, animals and microorganisms which usually have biological activities are defined as natural products. Natural products and their derivatives had played an important role of being a source of active ingredients for therapeutic drugs.[1]

In the ancient time, people had started to use local flora and fauna to treat various diseases. Some traditional healing systems, such as traditional Chinese medicine, Ayurveda, as well as western medicine, were developed at that time.[2] As a result, an increasing number of crude preparations were discovered to have medicinal effects.

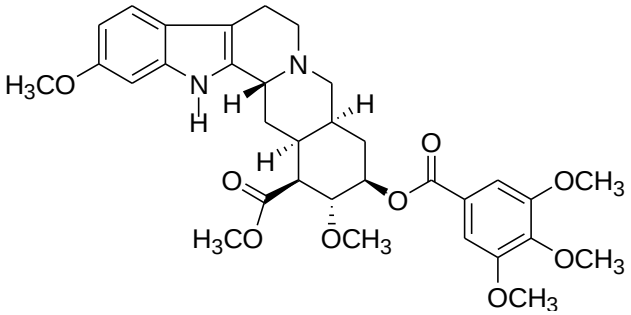
The chemical investigation of plants has started already a long time ago in medicinal research. The analgesic morphine (**1**) was first isolated in 1804 by Friedrich Sertürner, as the active principle in opium poppy known in ancient Egypt. In 1831, the German pharmacist Heinrich Mein first prepared crystallized atropine (**2**) from *Atropa belladonna* (Solanaceae).[3] In 1952, antihypertensive reserpine (**3**) was isolated from *Rauwolfia serpentine*, an Indian snakeroot used for the treatment of fever and snakebites, which significantly attracted scientists' attentions on natural products.[4] The isolation of anticancer drugs vinblastine (**4**) and vincristine (**5**), was independently reported from a Madagascar periwinkle, *Vinca rosea*, by Noble and Svoboda in 1958 and 1960.[5] In 1969, the well-known anticancer drug, taxol (**6**), was initially isolated from the Western Pacific Yew *Taxus brevifolia*, and it was reported to be beneficial for treatment of ovarian and breast cancer. In 1972, a research group led by Tu Youyou discovered artemisinin (**7**) from the leaves of *Artemisia annua*, which is effective in treating malaria.[6,7]



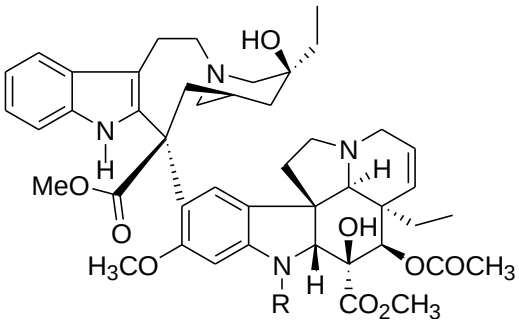
morphine (**1**)



atropine (**2**)

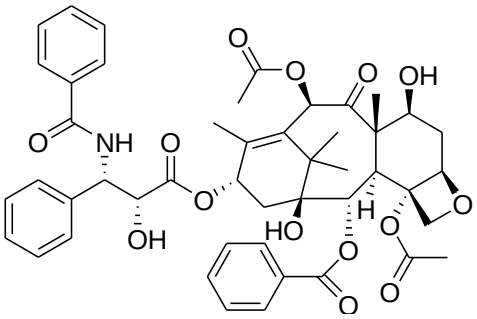


reserpine (**3**)

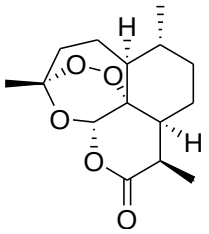


R = CH_3: vinblastine (**4**)

R = CHO: vincristine (**5**)



taxol (**6**)



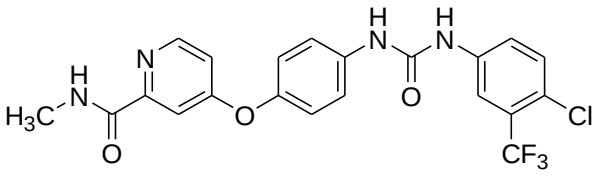
artemisinin (**7**)

Nowadays, scientists investigate the natural products for chemotherapeutic use from different natural sources: plant kingdom, microbial and marine world, animal sources, as well as venoms and toxins. A large variety of chemical constituents has been isolated from nature. Together with synthetically modified natural products, natural products have been developed to be suitable for the treatment of almost all human diseases.[8] According to a recent review analyzing the source of new drugs from 1981 to 2010, it is said that more than 50% of currently marketed drugs are actually natural products or derived from natural products.[9]

Despite the great success of natural products in drug discovery, classical natural product-based drug research has been gradually replaced by molecular target-based drug discovery. This decreased emphasis can be attributed to two major factors: the introduction of the approach of high-throughput screening (HTS) and the development of combinatorial chemistry.[9] The HTS process based on molecular targets can quickly test the bioactivity of numerous compound samples. However, most natural product programs could not meet this increased demand, not to mention crude natural product extracts are always complex mixtures with serious impurity influence. Combinatorial chemistry was also developed to offer more “drug-like” chemical compounds to provide the perfect hit in HTS efforts.

Although the approach involving the combination of HTS and combinatorial chemistry will no doubt create more opportunities for drug discovery, it is surprising that this approach delivered only one antitumor drug Nexavar (**8**) approved by FDA in 2005 for

renal carcinoma and in 2007 for hepatocellular carcinoma.[10]



Nexavar (**8**)

Clearly, drug discovery still faces a number of challenges. Nevertheless, a combination of modern natural product development and improved combinatorial chemistry linked with HTS will undoubtedly supply more unique and highly promising lead compounds for the drug discovery process.[11]

The typical process of discovering natural products as lead compounds, is bioassay-guided purification. A general scheme for the isolation of bioactive natural products and their development towards a drug candidate is shown in Figure 1.1.[1]

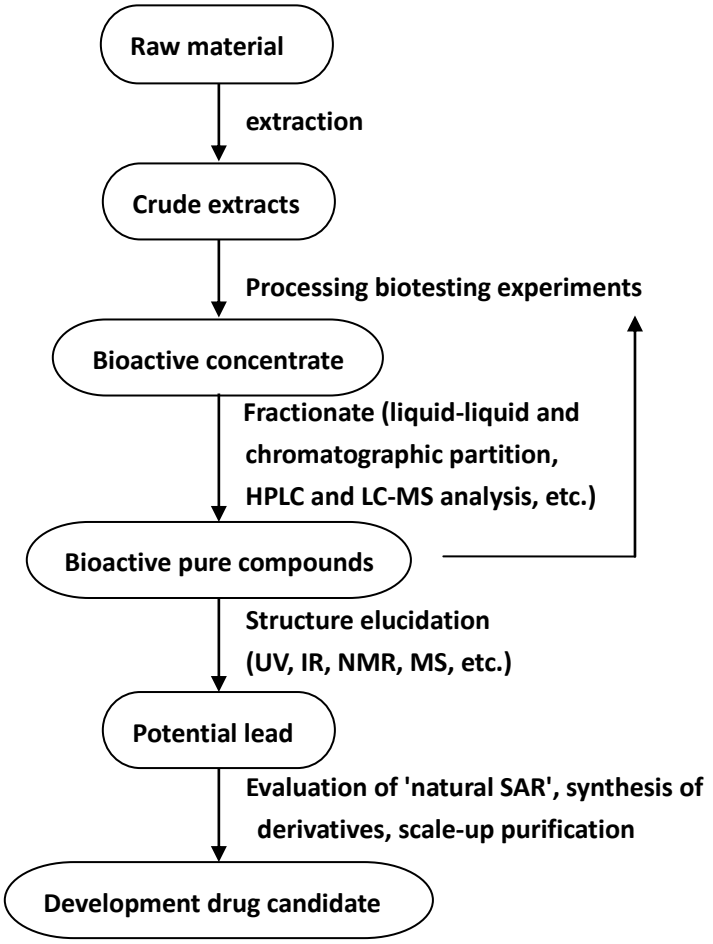


Figure 1.1 Chemical process for natural product drug discovery

1.2 Genus *Gynura* and overview of compounds isolated from *Gynura* Cass.

1.2.1 Description of the genus *Gynura*

Gynura species, belonging to the tribe Senecioneae in the family Asteraceae, are always succulent herbs, rarely shrubs (Figure 1.2). Their leaves grow alternately, in dentate or pinnate divided shape. They have typical discoid capitula, solitary or few to numerous corymbose in yellow or purple color. Involucres are campanulate or cylindric, with 9-13 uniseriate phyllaries and scarious margins. Receptacle is flat, areolate or shortly fimbriate. Anthers are entire or subauriculate at base. Style branches are slender and puberulent. Achenes are cylindric with ribs.[12]

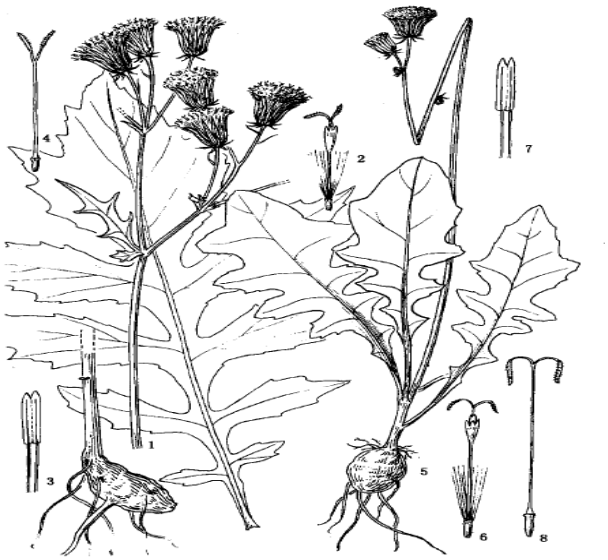


Figure 1.2 Representative *Gynura* species: *G. japonica* (1-4), *G. pseudochina* (5-8)

The genus *Gynura* comprises approximately 40 species mainly distributed in Asia, Africa and Australia, of which 10 species were recorded in the south of China. They are systematic classified as below:[12]

- ***Gynura barbareifolia*** Gagnepain
- ***Gynura bicolor*** (Roxburgh ex Willdenow) Candolle
- ***Gynura cusimbua*** (D. Don) S. Moore
- ***Gynura divaricata*** (Linnaeus) Candolle
- ***Gynura elliptica*** Y. Yabe & Hayata
- ***Gynura formosana*** Kitamura
- ***Gynura japonica*** (Thunberg) Juel
- ***Gynura nepalensis*** Candolle
- ***Gynura procumbens*** (Loureiro) Merrill
- ***Gynura pseudochina*** (Linnaeus) Candolle

1.2.2 Phytochemical investigation of plant species from the genus *Gynura*

Previous chemical investigations on *Gynura* Cass. demonstrated the presence of pyrrolizidine alkaloids, steroids, flavonoids, triterpenoids, chromone derivatives, cerebrosides and phenolics.[13-44] Besides, aliphatic compound derivatives, organic acids and other compounds were reported as well. All the compounds and their origin are listed in Table 1.1 and their chemical structures are shown as below.

Table 1.1 Overview of natural products isolated from *Gynura* Cass.

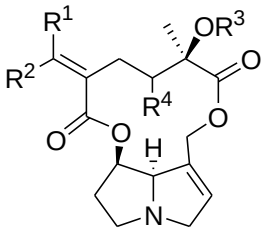
Compound name	Plant origin	Reference
1. Pyrrolizidine alkaloids		
1.1 Retronecine type		
senecionine (**9**)	*G. segetum*	13-17
	G. sarmentosa	18
	G. elliptica	19
gynuramine (**10**)	*G. scandens*	20
acetylgynuramine (**11**)	*G. scandens*	20,21
integerrimine (**12**)	*G. divaricata*	22
usaramine (**13**)	*G. divaricata*	22
jacobine (**14**)	*G. crepidioides*	23
jacoline (**15**)	*G. crepidioides*	23
seneciphylline (**16**)	*G. segetum*	16
seneciphyllinine (**17**)	*G. segetum*	16
spartioidine (**18**)	*G. segetum*	16
1.2 Otonecine type		
otosenine (**19**)	*G. sarmentosa*	17
senkirkine (**20**)	*G. sarmentosa*	17
	G. elliptica	19
2. Steroids		
3-epi-diosgenin 3-O-β-D-glucopyranoside (**21**)	*G. japonica*	24
3-epi-ruscogenin (**22**)	*G. japonica*	24
3-epi-sceptrumgenin 3-O-β-D-glucopyranoside (**23**)	*G. japonica*	24
3-epi-neoruscogenin (**24**)	*G. japonica*	24
β-sitosterol 3-O-β-D-glucopyranoside (**25**)	*G. procumbens*	25
	G. formosana	26
	G. japonica	27
	G. divaricata	28
stigmasterol 3-O-β-D-glucopyranoside (**26**)	*G. procumbens*	25
	G. formosana	26
	G. divaricata	28
	G. japonica	27
β-sitosterol (**27**)	*G. procumbens*	25
	G. formosana	26
	G. divaricata	28,29
	G. bicolor	30

	G. japonica	27
stigmasterol (**28**)	*G. procumbens*	25
	G. formosana	26
	G. divaricata	28,29
	G. japonica	27
β-sitosterone (**29**)	*G. japonica*	27
stigmasterone (**30**)	*G. japonica*	27
(22*E*,24*S*)-7α-hydroperoxystigmasta-5,22-dien-3β-ol (**31**)	*G. japonica*	27
(24*R*)-7α-hydroperoxystigmasta-5,22-dien-3β-ol (**32**)	*G. japonica*	27
(22*E*,24*S*)-stigmasta-1,4,22-trien-3-one (**33**)	*G. japonica*	27
(22*E*,24*S*)-stigmasta-1,4-dien-3-one (**34**)	*G. japonica*	27
3. Flavonoids		
quercetin (**35**)	*G. divaricata*	29,31
	G. bicolor	32
quercetin 3-O-β-D-glucopyranoside (isoquercitin, **36**)	*G. divaricata*	29
	G. bicolor	32
quercetin 3-O-α-L-rhamnopyranoside (**37**)	*G. bicolor*	32
quercetin 3-O-rutinoside (rutin, **38**)	*G. procumbens*	33,34
	G. formosana	35,36
	G. divaricata	31,29
	G. bicolor	30,32
	G. pseudochina	37
quercetin 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**39**)	*G. procumbens*	33,34
kaempferol (**40**)	*G. divaricata*	31
	G. bicolor	30,32
kaempferol 3-O-β-D-glucopyranoside (astragalin, **41**)	*G. divaricata*	31
	G. procumbens	33,34
	G. bicolor	30
kaempferol 3-O-rutinoside (**42**)	*G. procumbens*	33,34
	G. formosana	35,36
	G. divaricata	29
	G. bicolor	30
kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**43**)	*G. formosana*	36
kaempferol 3-O-robinobioside (**44**)	*G. formosana*	35
kaempferol 3,7-di-O-β-D-glucopyranoside (**45**)	*G. divaricata*	31
kaempferol 3-O-rutinoside-7-O-β-D-glucopyranoside (**46**)	*G. divaricata*	31
homoplantaginin (**47**)	*G. bicolor*	30
anthocyanin (**48**)	*G. bicolor*	18
	G. aurantiaca cv.	18

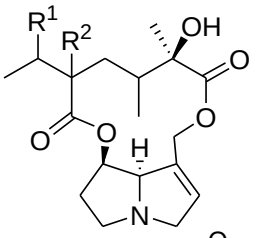
4.	**Triterpenoids**		
	α-amyrin (**49**)	*G. formosana*	38
		G. bicolor	30
	β-amyrin (**50**)	*G. formosana*	38
		G. bicolor	30
	β-amyrin 3-O-β-D-glucopyranoside (**51**)	*G. bicolor*	30
	3-epi-friedelanol (**52**)	*G. formosana*	26
		G. divaricata	29
		G. bicolor	30
	3-*O*-acetyl-epi-friedelanol (**53**)	*G. formosana*	26
		G. divaricata	29
	friedelin (**54**)	*G. formosana*	26
		G. divaricata	28
	isobauerenone (**55**)	*G. formosana*	26
	isobauerenol (**56**)	*G. formosana*	26
	glutinol (**57**)	*G. formosana*	26
	α-tocospirone (**58**)	*G. japonica*	27
5.	**Chromone derivatives**		
	6-acetyl-2-hydroxymethyl-2-methylchroman-4-one (**59**)	*G. formosana*	38,39
		G. japonica	27
	6-acetyl-2,2-dimethylchroman-4-one (**60**)	*G. elliptica*	19
		G. japonica	27
	6-hydroxy-2,2-dimethylchroman-4-one (**61**)	*G. elliptica*	19
	gynunone (**62**)	*G. elliptica*	19
	gynunol (**63**)	*G. elliptica*	19
	gynuraone (**64**)	*G. japonica*	27
6.	**Cerebrosides**		
	gynuramides 1-4 (**65-68**)	*G. japonica*	40
	1-O-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,10*E*)-2-[(2′*R*)-2′-hydro-xyltricosanoylamino]-10-octadecene-1,3,4-triol (**69**)	*G.divaricata*	41
	1-O-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,10*Z*)-2-[(2′*R*)-2′-hydro-xyltricosanoylamino]-10-octadecene-1,3,4-triol (**70**)	*G.divaricata*	42
	1-O-β-D-glucopyranosyl-(2*S*,3*S*,4*R*,10*E*)-2-[(2′*R*)-2′-hydro-xyldocosanoylamino]-10-octadecene-1,3,4-triol (**71**)	*G.divaricata*	43
7.	**Phenolics**		
	3,5-di-caffeoylquinic acid (**72**)	*G. pseudochina*	37
		G.divaricata	31
	4,5-di-caffeoylquinic acid (**73**)	*G. pseudochina*	37
	5-caffeoylquinic acid (chlorogenic acid, **74**)	*G. pseudochina*	37
	oleuropein (**75**)	*G. aurantiaca*	44
	caffeic acid (**76**)	*G. formosana*	35,36
	syringaldehyde (**77**)	*G. elliptica*	19
	benzoic acid (**78**)	*G. japonica*	27
	4-hydroxybenzoic acid (**79**)	*G. bicolor*	30

vanillin (**80**)	*G. elliptica*	19
	G. japonica	27
8. Terpenylcoumarin		
gynurone (**81**)	*G. crepidioides*	45
9. Aliphatic compound derivatives		
1-tridecene-3,5,7,9,11-pentayne (**82**)	*G. crepidioides*	45
n-eicosane (**83**)	*G. divaricata*	28
methyl palmitate (**84**)	*G. formosana*	46
methyl linoleate (**85**)	*G. formosana*	46
methyl oleate (**86**)	*G. formosana*	46
octadecan-1-ol (**87**)	*G. bicolor*	30
docosan-1-ol (**88**)	*G. formosana*	46
tetracosan-1-ol (**89**)	*G. divaricata*	28
octacosan-1-ol (**90**)	*G. divaricata*	28
dotriacontan-1-ol (**91**)	*G. formosana*	26
2,3-dihydroxypropyl palmitate (**92**)	*G. formosana*	46
3(20)-phytene-1,2-diol (**93**)	*G. formosana*	46
10. Organic acids		
succinic acid (**94**)	*G. segetum*	15
palmitic acid (**95**)	*G. divaricata*	28
	G. japonica	27
hexacosanoic acid (**96**)	*G. japonica*	27
octacosanoic acid (**97**)	*G. divaricata*	28
11. Pyrimidines		
thymine (**98**)	*G. segetum*	15
uracil (**99**)	*G. formosana*	38
12. Purine		
adenine (**100**)	*G. segetum*	15
adenosine (**101**)	*G. divaricata*	29
uridine (**102**)	*G. divaricata*	29
13. Others		
pheophorbide a (**103**)	*G. formosana*	46
pheophorbide b (**104**)	*G. formosana*	46
caryophyllene oxide (**105**)	*G. japonica*	27
2,6-dimethoxy-1,4-benzoquinone (**106**)	*G. japonica*	27
D-mannitol (**107**)	*G. segetum*	15

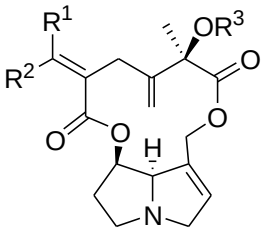
Pyrrolizidines



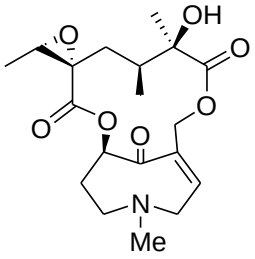
9: R^1 = H, R^2 = R^3 = R^4 = CH_3
10: R^1 = H, R^2 = R^3 = CH_3, R^4 = CH_2OH
11: R^1 = H, R^2 = R^3 = CH_3, R^4 = CH_2OAc
12: R^1 = R^3 = R^4 = CH_3, R^2 = H
13: R^1 = R^4 = CH_3, R^2 = H, R^3 = CH_2OH



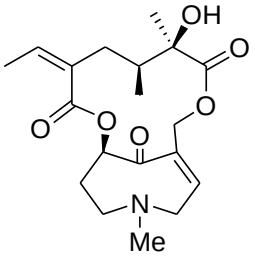
14: $R^1C—CR^2$ = 
15: R^1 = R^2 = OH



16: R^1 = R^3 = H, R^2 = CH_3
17: R^1 = H, R^2 = CH_3, R^3 = Ac
18: R^1 = CH_3, R^2 = R^3 = H

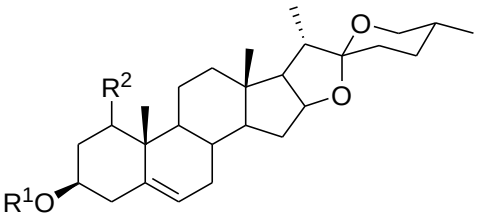


19

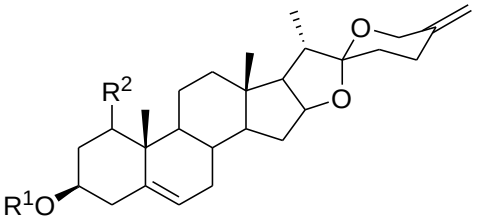


20

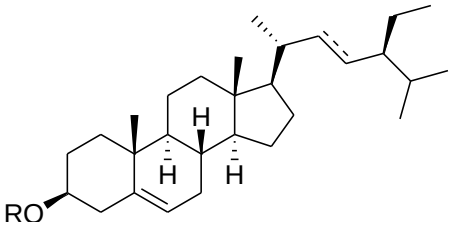
Steroids



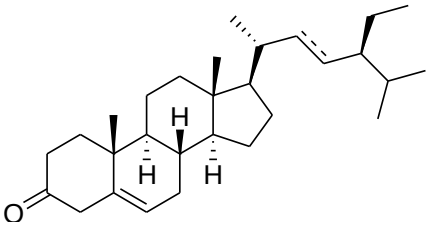
21: R^1 = β-D-glucopyranosyl, R^2 = H
22: R^1 = H, R^2 = OH



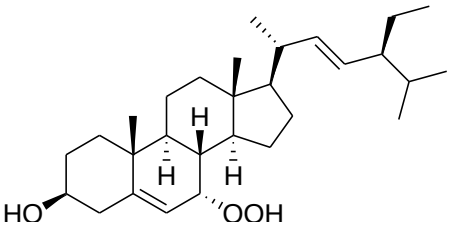
23: R^1 = β-D-glucopyranosyl, R^2 = H
24: R^1 = H, R^2 = OH



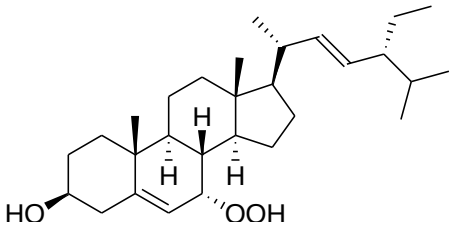
25: R = β-D-glucopyranosyl
26: R = β-D-glucopyranosyl, Δ22
27: R = H
28: R = H, Δ22



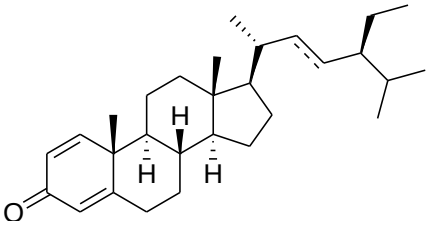
29
30: Δ22



31



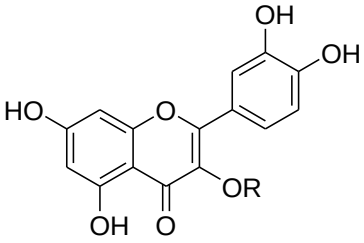
32



33: Δ22

34

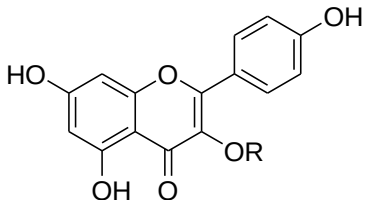
Flavonoids



35: R = H

36: R = β-D-glucopyranosyl

37: R = α-L-rhamnopyranosyl



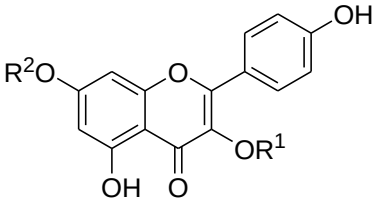
40: R = H

41: R = β-D-glucopyranosyl

42: R = rutinosyl

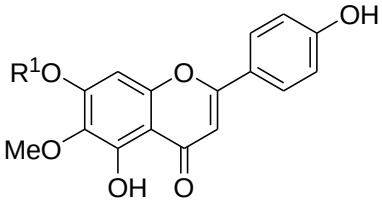
38: R = rutinosyl **43**: R = α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl

39: R = α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranosyl **44**: R = robinobiosyl

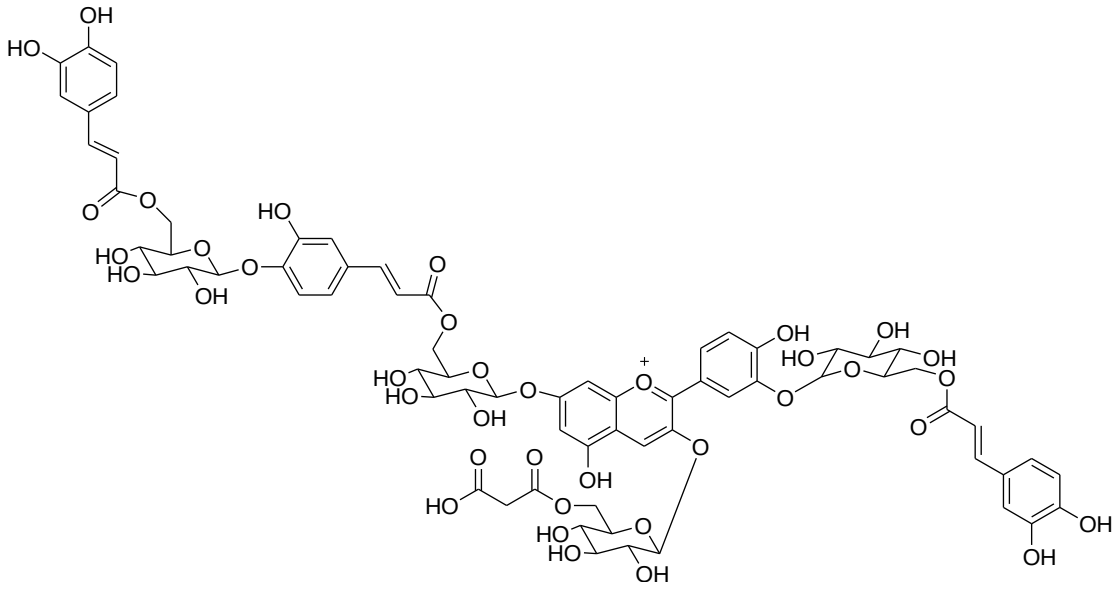


45: R^1 = β-D-glucopyranosyl, R^2 = β-D-glucopyranosyl

46: R^1 = rutinosyl, R^2 = β-D-glucopyranosyl

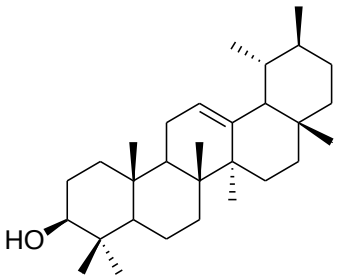


47: R^1 = β-D-glucopyranosyl

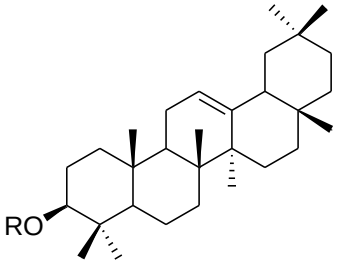


48

Triterpenoids

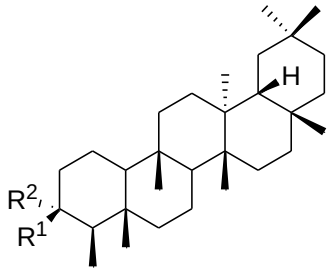


49



50: R = H

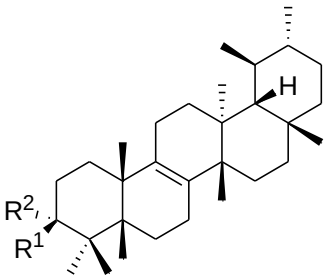
51: R = β-D-glucopyranosyl



52: R^1 = OH, R^2 = H

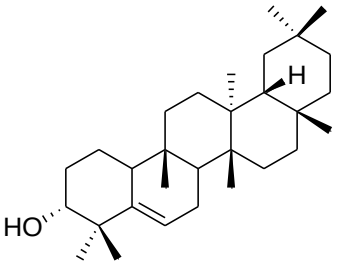
53: R^1 = OAc, R^2 = H

54: CR^1R^2 = C(=O)

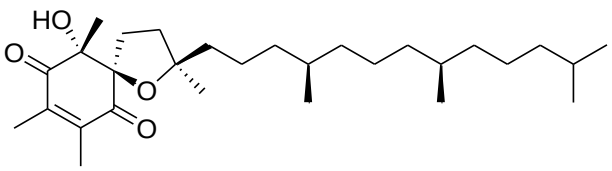


55: CR^1R^2 = C(=O)

56: R^1 = OH, R^2 = H

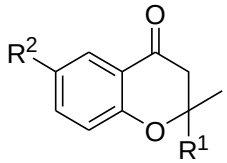


57



58

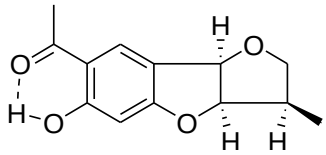
Chromone derivatives



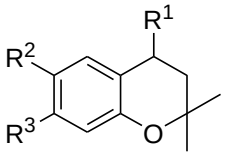
59: $R^1 = CH_2OH$, $R^2 = COCH_3$

60: $R^1 = Me$, $R^2 = COCH_3$

61: $R^1 = Me$, $R^2 = OH$



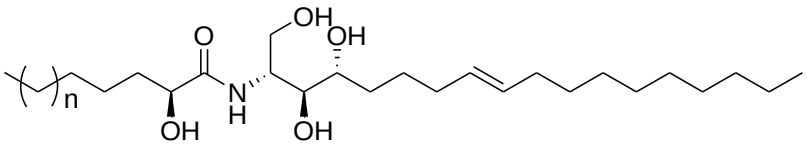
62



63: $R^1 = R^2 = OH$, $R^3 = H$

64: $R^1 = R^3 = OH$, $R^2 = H$

Cerebrosides

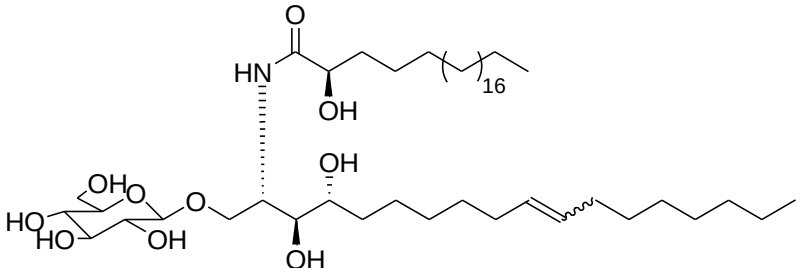


65: n = 19

66: n = 18

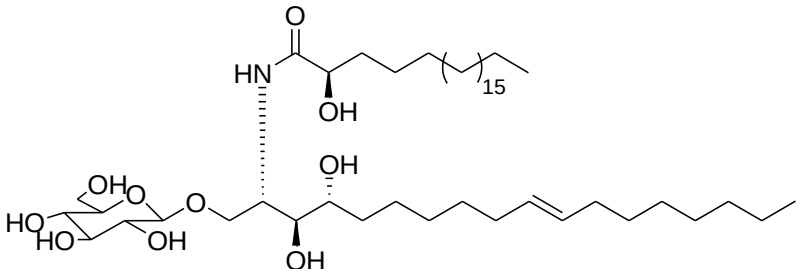
67: n = 17

68: n = 16



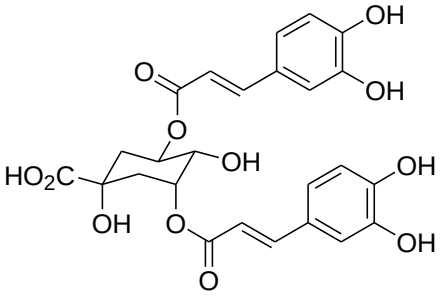
69: 22-E type

70: 22-Z-type

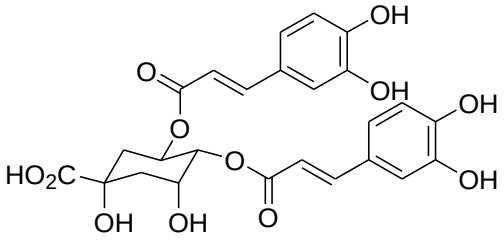


71

Phenolics



72



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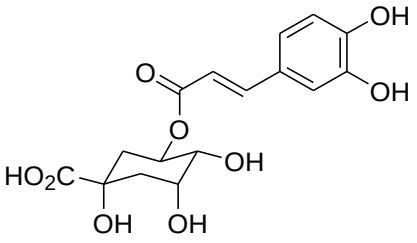
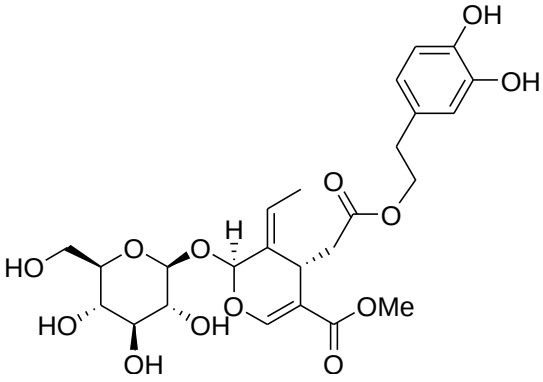
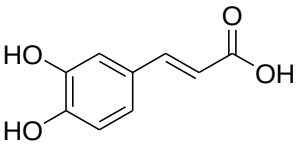


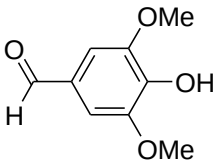
74



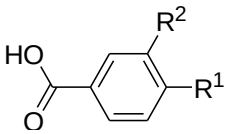
75



76



77

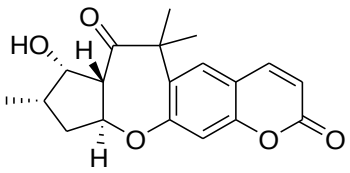


78: $R^1 = R^2 = H$

79: $R^1 = OH$, $R^2 = H$

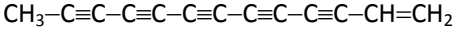
80: $R^1 = OH$, $R^2 = OMe$

Terpenylcoumarin



81

Aliphatic compound derivatives



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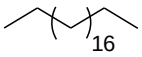
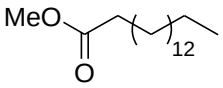
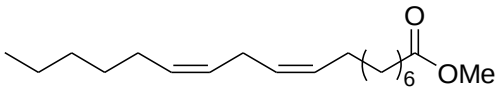


83



84



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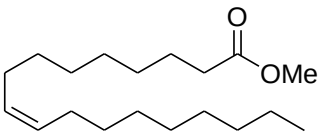
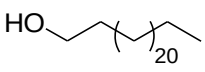
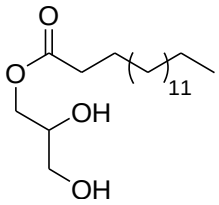


86



87: n = 14
88: n = 18
89: n = 20
90: n = 24
91: n = 28



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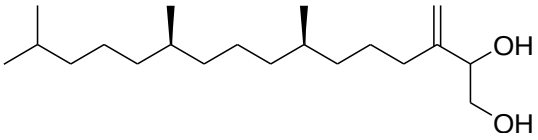
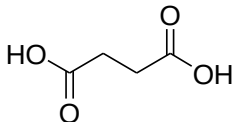
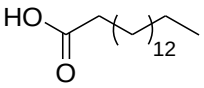


93

Organic acids



94



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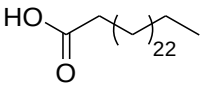

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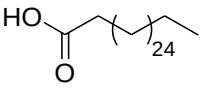
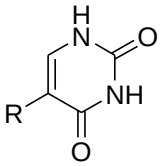


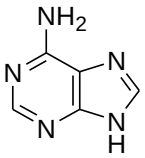
97

Pyrimidines



98: R = CH_3
99: R = H

Purines



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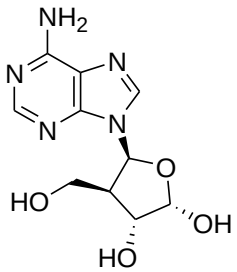

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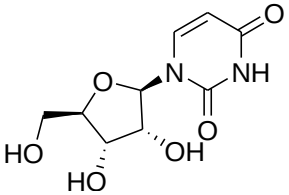
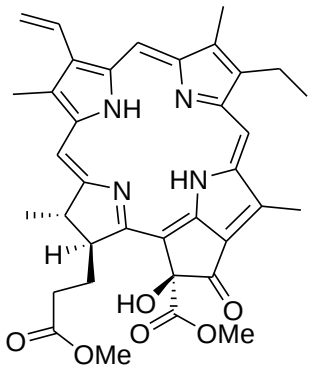


102

Others



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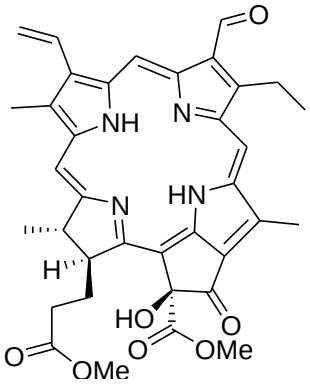

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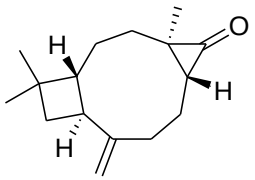
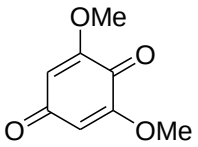


105



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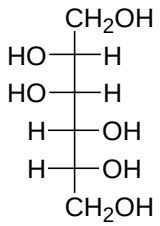


107

1.3 Natural Flavonoids as Potential Herbal Medication for the Treatment of Diabetes Mellitus and Its Complications

Diabetes mellitus, followed by its various complications, is becoming a serious threat to human health in the world. The development of antidiabetic medication from natural products, especially those originated from plants with a traceable folk-usage history in treating diabetes, is receiving more attention. Many studies highlighted not only the benefits of natural flavonoids with hypoglycemic effects, but also their importance in the management of diabetic complications. This part describes selected natural flavonoids (active natural components) that have been validated for their hypoglycemic properties, together with their mechanisms of action. Also their activities in the treatment of diabetes complications demonstrated via laboratory diabetic animal models, in vitro and in clinical trials using human subjects, are discussed. Published papers from 2000 to date on flavonoids and diabetes were covered through accessing databases of Web of Science and PubMed. The major potential benefits of natural flavonoids discussed here clearly suggest that these substances are lead compounds with sufficient structural diversity of great importance in the antidiabetic drug developing process.

1.3.1 Introduction

Diabetes mellitus is a metabolic disorder disease with hyperglycemia (high blood glucose) as its most typical character. This hyperglycemia results in three classical symptoms of diabetes: polyuria, polydipsia and polyphagia. The World Health Organization divides diabetes mellitus into three main types: Type 1, Type 2 and gestational diabetes. Type 1

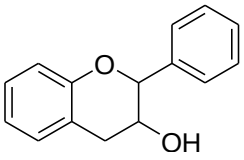
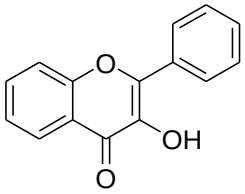
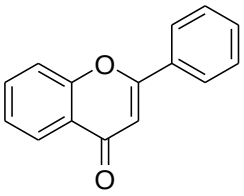
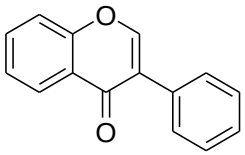
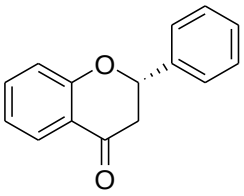
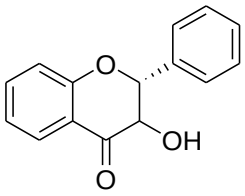
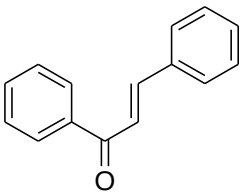
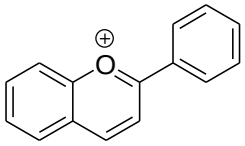
diabetes (formerly known as Insulin-Dependent Diabetes Mellitus) results from an insulin production problem, which requires lifelong administration of insulin. Type 2 diabetes (formerly called Non-Insulin-Dependent Diabetes Mellitus) is caused by the body's defect in insulin response, called insulin resistance. Type 2 diabetes comprises 90% of people with diabetes around the world, mainly due to obesity and physical inactivity. Gestational diabetes is hyperglycemia with onset or first recognition during pregnancy.[47]

Data from the International Diabetic Federation (IDF) demonstrates that the number of people with diabetes in 2011 has reached 366 million, representing 5.23% of the world's population.[48] With 90 million adults with this disease, China has become the country with the largest population of diabetics in the world. According to the IDF, diabetes and its complications have a significant economic impact on medical expenditure, which already accounts for 5-18% of the total healthcare budget in many countries.

With a centuries-old history till now, humans use natural substances, mostly by means of a plant extract or a crude medical recipe, to cure diseases. Many of them serve as leads of important drugs that have become mainstays in modern therapy.[49] For example, metformin, belonging to a biguanide class of antidiabetic drugs, is originally from *Galega officinalis*, a plant used in folk medicine.[50] Acarbose, the most widely used α-glucosidase inhibitor for the treatment of Type 2 diabetes, resulted from a screening performed by Bayer AG of natural products isolated from *Actinomycetes* species for inhibition of digestive enzymes.[51] Natural products are an abundant source for antidiabetic drug discovery despite the lack of content determination, quality and safety control. By following an integrated approach and using correct procedures, antidiabetic agents from medicinal plants especially used in folk medicine for diabetes treatment may provide an alternative to the classical drugs used (insulin and oral hypoglycemic agents), with few side effects and low cost for the maintenance of treatment.[52]

Flavonoids are a class of plant secondary metabolites with the backbone of flavone (2-phenylchromen-4-one). Important subgroups are flavanols, flavonols, flavones, isoflavones, flavanones, flavanonols, chalcones and anthocyanins, which are defined by the functional groups attached to the basic flavonoid structure (Table 1.2). They have a broad range of biological activities and numerous studies have been carried out on their potential role in the treatment of diabetes and its complications. This overview gathers information on the in vitro and in vivo bioassay of plants containing flavonoids used in the treatment of conditions consistent with diabetes symptomatology. It summarizes the progress in natural flavonoid research, emphasized on the treatment of diabetes and its complications. Because of the multifactorial pathogenicity of diabetes, information on the mechanism of action of natural flavonoids in the treatment of diabetes is presented at the beginning.

Table 1.2 Flavonoid subgroups and their structures

Group	Structure	Group	Structure
flavanol		flavonol	
flavone		isoflavone	
flavanone		flavanonol	
chalcone		anthocyanin	

1.3.2 Mechanisms of action of natural flavonoids for the treatment of diabetes mellitus

Currently, insulin injection and oral administration of antidiabetic drugs are the mainstay of available therapy for the treatment of diabetes mellitus. Antidiabetic drugs in the western market can be classified as sensitizers, secretagogues, insulin analogues, α-glucosidase inhibitors, amylin analogues and others, which are used as monotherapy or in combination by the patients whose disease is more serious for a better glycemic regulation.[53] Unfortunately, most of them have prominent side effects and, moreover, lack the ability to prevent the development of diabetic complications. Worldwide, pharmacologists are devoted to the search for alternative therapeutic approaches for this metabolic disease.[54]

Up to now, more and more plants used as ethnopharmacological antidiabetic remedies are being confirmed, and their responsible chemical profiles for the hypoglycemic effect are being studied. Moreover, the mechanism of action of these substances is being elucidated in a meticulous way. The main mechanisms of action of natural flavonoids can be described as below, and information of the corresponding flavonoids is listed in Table 1.3.

1.3.2.1 Action on islet β-cells and release of insulin from β-cells

When blood sugar concentration rises, β-cells of the endocrine pancreas release insulin,

the hormone that controls the level of glucose, into the blood stream.[55] Decreased viability and dysfunction of pancreatic β-cells leads to the development of diabetes as critical metabolic disorder.

Many flavonoids were proposed as potential antidiabetic agents because they exert multiple actions on islet β-cells. In this respect, the functions of flavonoids can be divided into (i) protection against β-cell damage,[56,57,58] (ii) increased proliferation of islet β-cells[59] and (iii) preservation of insulin signaling by increased insulin secretion.[60-63]

1.3.2.2 Enhancement of glucose utilization in tissues and organs

Diabetes is a disease with disorders in glucose metabolism, which means the deficiency of glucose utilization, inducing an increased glucose output. Glucose utilization involves the cellular glucose transport, which is mediated by the family of facilitative glucose transporters (GLUTs) as solute carriers. This process occurs through a signaling pathway involving the insulin receptor tyrosine kinase.[64] In diabetes, this complex system of insulin-stimulated whole-body glucose utilization is impaired, probably because of defective GLUT-4 translocation and aberrant insulin signal transduction.[65]

It has been proven that several naturally occurring flavonoids affect this transduction and enhance the utilization of glucose, by their action on glucose transport[66-69] and insulin-receptor signaling.[70-72]

1.3.2.3 Reduction of breakdown of glycogen

In the course of consumption, glucose is phosphorylated to glucose-6-phosphate by glucokinase, followed by glycogen synthesis, glycolysis or triglyceride synthesis.[73] Insulin inhibits gluconeogenesis and glycogenolysis by its effect on regulating the transcription of genes encoding hepatic and muscular enzymes, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (G-6-Pase).[74]

Flavonoids have been found to increase the glucokinase level, thus promoting glycogen synthesis,[56,75] to reduce G-6-Pase and phosphoenolpyruvate carboxykinase gene expression, thus inhibiting gluconeogenesis or glycogenolysis,[76-79] or both.[80-83]

1.3.2.4 Inhibition of α-glucosidase to reduce intestinal glucose absorption

Carbohydrates are hydrolyzed to their corresponding monosaccharides by enzymes present in the brush border of small intestine before absorption. Among these enzymes, α-glucosidase is the most important enzyme in carbohydrate digestion. Acarbose, a popular antidiabetes drug, working as α-glucosidase inhibitor, can significantly decrease the Type 2 diabetics' fasting and postprandial blood glucose, glycosylated hemoglobin and improve insulin sensitivity effectively.[84,85]

Many flavonoids can be used as α-glucosidase inhibitors,[86-92] and they can prevent the digestion of carbohydrates and delay the glucose absorption. Hence, α-glucosidase inhibitors reduce the impact of carbohydrates on blood sugar. Besides, sucrase, maltase

and α-amylase inhibitors[86,93,94] also show hypoglycemic potential in the treatment of diabetes mellitus.

1.3.2.5 Antioxidant free radical scavenging activity

The cytotoxic action of alloxan and streptozotocin (STZ), which are mostly used to build up diabetes mellitus in experimental animals, is mediated by reactive oxygen species (ROS).[95] Diabetics and experimental animal models exhibit high oxidative stress, and it has been suggested to be one factor on the development of diabetes related complications.

Flavonoids are well-known for their function as natural antioxidant agents by scavenging reactive oxygen species, by virtue of the number and arrangement of their phenolic hydroxyl groups attached to aromatic ring structures.[96] The protective effects of flavonoids in diabetes as antioxidants are often ascribed to their capacity to scavenge free radicals in vitro,[97-100] to activate antioxidant enzymes,[101] and to increase non-enzymic antioxidants.[102]

1.3.2.6 Inhibition of AGE formation and AGE-mediated damage

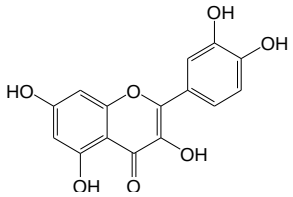
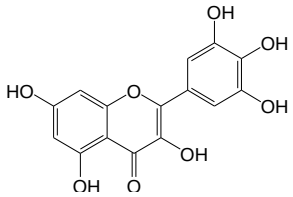
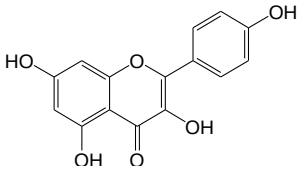
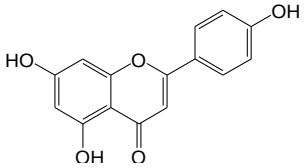
Advanced glycation end-products (AGEs) are the result of a chain of chemical reactions initiated by a glycation reaction (Maillard reaction). An abnormally elevated blood glucose level in diabetes mellitus causes the formation of AGEs in vivo, which is implicated in the development of chronic diabetic complications.[103]

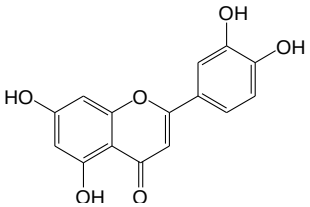
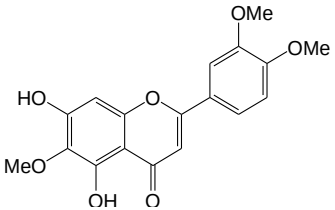
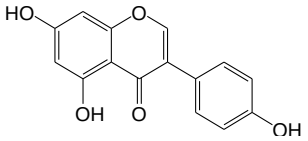
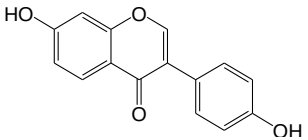
Suppression of the formation of Maillard reaction products by dietary phenolic compounds has also been shown in food model systems. Structure-activity studies of flavan-3-ols on the formation of Maillard reaction products in a glucose/glycine model system showed that the main mechanism by which these flavanols reduced product formation was the trapping of reactive carbonyl compounds, formed from sugar fragmentation.[104]

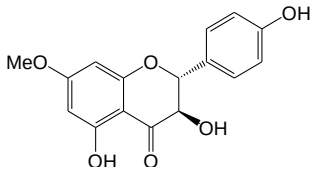
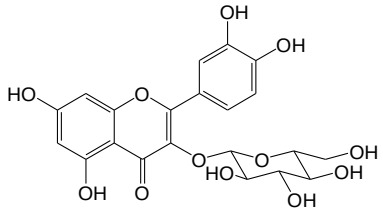
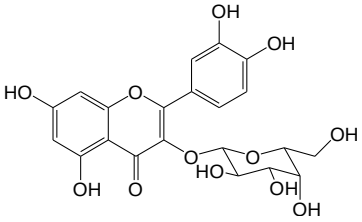
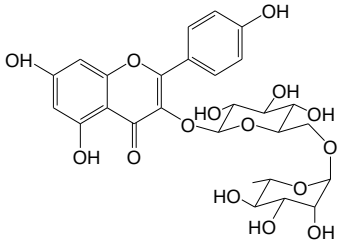
Moreover, many studies have demonstrated that free radicals such as hydrogen peroxide, superoxide anion radicals and singlet oxygen, participate in the formation of AGEs. Those substances with outstanding antioxidant activity or/and free radical scavenging properties inhibit the formation of AGEs.[105] Flavonoids possess additional properties relevant to prevent diabetic complications with their potent antioxidant abilities, by providing a protective effect against hyperglycemia-mediated protein damage.[106]

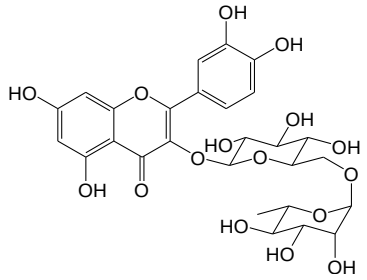
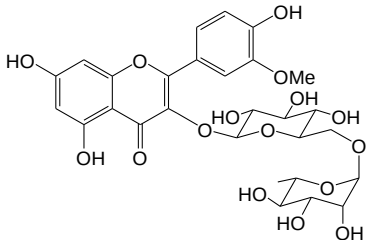
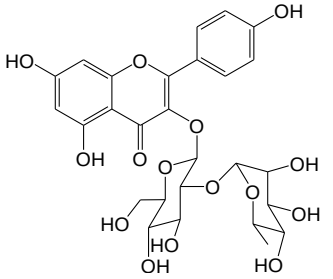
There are increasing reports on natural products with combined antiglycation, antioxidant properties and reduced toxicity.[107] As such, a number of plant-derived flavonoids[105,108] and a purified micronized flavonoid fraction[109] have been reported to inhibit glycation.

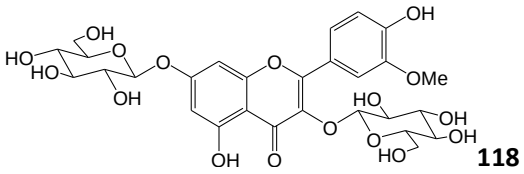
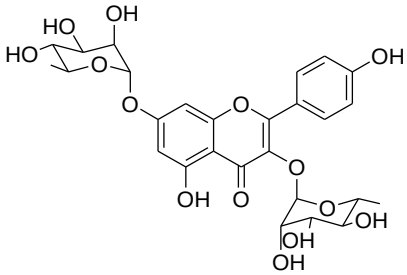
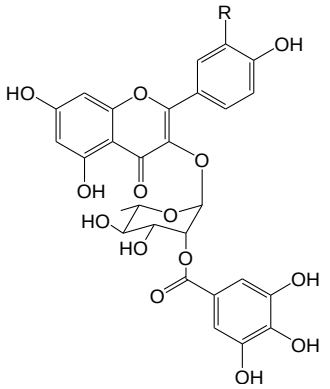
Table 1.3 Mechanisms of action of natural flavonoids for the treatment of diabetes mellitus

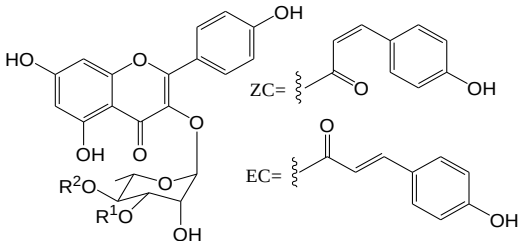
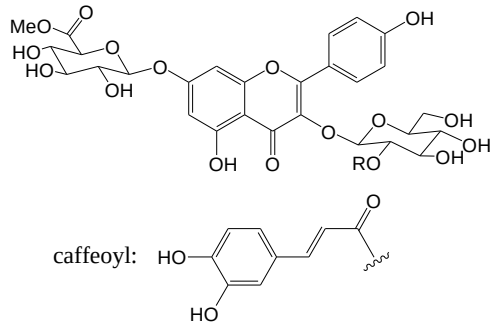
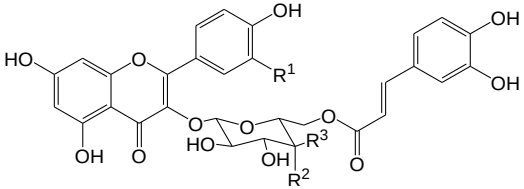
Flavonoid	Category	Structure	Mechanism	Literature reference
Quercetin (**35**)	flavonols	 **35**	promotion of glycogen synthesis; protection of β-cells against damage; inhibition of α-glucosidase	[56]; [56-58]; [89]
Myricetin (**108**)	flavonols	 **108**	activation of glucose transport; stimulation of glucose uptake; promotion of glycogen synthesis; inhibition of sucrase, maltase and α-amylase	[67]; [71]; [75]; [94]
Kaempferol (**40**)	flavonols	 **40**	inhibition of sucrase, maltase and α-amylase	[94]
Apigenin (**109**)	flavones	 **109**	scavenging of free radicals	[100]

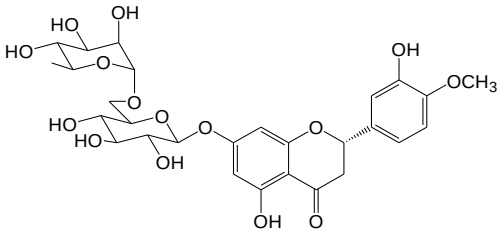
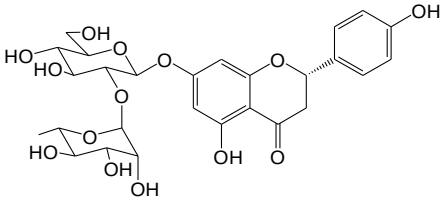
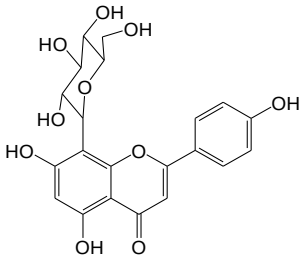
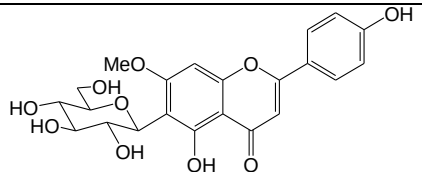
Luteolin (**110**)	flavones	 **110**	inhibition of both α-glucosidase and α-amylase	[86]
Eupatilin (**111**)	flavones	 **111**	enhancement of pancreatic β-cell function to increase insulin	[62]
Genistein (**112**)	isoflavones	 **112**	proliferation of islet β-cells; stimulation of insulin secretion in pancreatic β-cells; inhibition of glucose-6-phosphatase; reduction of breakdown of glycogen	[59]; [61]; [79]; [82]
Daidzein (**113**)	isoflavones	 **113**	stimulation of glucose uptake; reduction of breakdown of glycogen	[67]; [72]

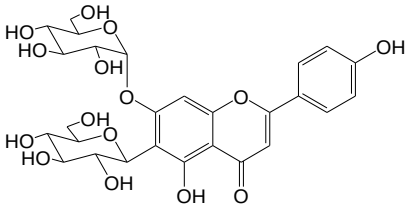
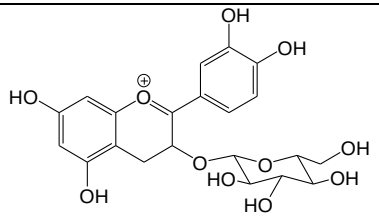
7-O-Methylaromadendrin (**114**)	flavanonols	 **114**	stimulation of glucose uptake	[72]
Isoquercitin (**36**)	flavonol glycosides	 **36**	inhibition of α-glucosidase; inhibition of advanced glycation endproduct formation	[88, 89]; [105]
Hyperin (**115**)	flavonol glycosides	 **115**	inhibition of advanced glycation endproduct formation	[105], [108]
Kaempferol 3-O-rutinoside (**42**)	flavonol glycosides	 **42**	inhibition of α-glucosidase	[92]

Rutin (**38**)	flavonol glycosides	 **38**	inhibition of α-glucosidase; reduction of breakdown of glycogen; activation of signaling in pancreatic β-cells; scavenging of free radicals; activation of antioxidant enzymes; increase non-enzymic antioxidants	[89], [105]; [83]; [63]; [100]; [101]; [102]
Isorhamnetine 3-O-rutinoside (**116**)	flavonol glycosides	 **116**	inhibition of α-glucosidase	[88]
Kaempferol 3-neohesperidoside (**117**)	flavonol glycosides	 **117**	stimulation of glucose uptake	[70]

Isorhamnetin 3,7-di-O-β-D-glucopyranoside (**118**)	flavonol glycosides	 **118**	scavenging of free radicals	[98]
Kaempferitrin (**119**)	flavonol glycosides	 **119**	scavenging of free radicals; stimulation of glucose uptake	[99]; [68]
Quercetin 3-O-α-(2''-galloyl)-rhamnoside (**120**) kaempferol 3-O-α-(2''-galloyl)-rhamnoside (**121**)	flavonol glycosides	 **120**: R = OH **121**: R = H	inhibition of glucose-6-phosphatase	[77]

Kaempferol 3-O-α-L-rhamnopyranoside (3"*E*,4"*E*)-di-*p*-coumaric acid ester (**121**), kaempferol 3-O-α-L-rhamnopyranoside (3"*E*, 4"*Z*)-di-*p*-coumaric acid ester (**122**)	flavonol glycosides	 **121**: $R^1 = R^2 =$ EC **122**: $R^1 =$ EC, $R^2 =$ ZC	inhibition of α-glucosidase	[87]
Corchoruside A (**123**), corchoruside B (**124**)	flavonol glycosides	 **123**: R = caffeoyl **124**: R = H	inhibition of α-glucosidase	[90]
Quercetin 3-O-(6-O-caffeoyl)-β-galactoside (**125**), kaempferol 3-O-(6-O-caffeoyl)-β-galactoside (**126**), kaempferol 3-O-6-O-caffeoyl)-β-glucoside (**127**)	flavonol glycosides	 **125**: $R^1 = R^2 =$ OH, $R^3 =$ H **126**: $R^1 = R^3 =$ H, $R^2 =$ OH **127**: $R^1 = R^2 =$ H, $R^3 =$ OH	inhibition of maltase	[93]

Hesperidin (**128**)	flavanone glycosides	 **128**	inhibition of glucose-6-phosphatase; reduction of breakdown of glycogen	[78]; [81]
Naringin (**129**)	flavanone glycosides	 **129**	inhibition of glucose-6-phosphatase; reduction of breakdown of glycogen	[78]; [81]
Vitexin (**130**)	flavone C-glycosides	 **130**	inhibition of α-glucosidase	[88]
Swertisin (**131**)	flavone C-glycosides	 **131**	inhibition of α-glucosidase	[88]

Apigenin 6-C-glucosyl-7-O-glucoside (**132**)	flavones C-glycoside	 **132**	inhibition of α-glucosidase	[91]
Cyanidin 3-O-glucoside (**133**)	anthocyanins	 **133**	inhibition of advanced glycation endproduct formation	[108]

1.3.3 Natural flavonoids with antidiabetic activities reported from in vivo studies

Besides the studies on the mechanisms of action of flavonoids against diabetes, attention was also given to their activities in the treatment of experimental diabetic animals. New studies on natural flavonoid compounds with diverse skeletons and antidiabetic potential reported since 2000 to date are presented here, with the parameters tested (Table 1.4).

Treatments with quercetin (**35**) were reported to significantly and dose-dependently decrease the plasma glucose, cholesterol and triglyceride (TG) level and to normalize the glucose tolerance curves of STZ-induced diabetic rats.[56] In a similar diabetic model, quercetin (**35**) administration resulted in a decrease in serum glucose and an increase in serum insulin levels. A preservation of islet cells was observed as well. In addition, by reducing plasma malondialdehyde (MDA), a marker of lipid peroxidation, the quercetin (**35**) treatment could reduce STZ-induced oxidative stress.[57,58]

In a study on treatment with rutin (**38**), the glycoside of quercetin (**35**), parameters such as plasma glucose and glycosylated hemoglobin (HbA1c) were found to be significantly reduced, together with an increase of insulin, C-peptide, hemoglobin and protein levels in STZ-induced diabetic Wistar rats. Treatment of diabetic rats with rutin (**38**) also resulted in a prevention of oxidative stress, since a decrease of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides and an increase of non-enzymatic antioxidants were detected.[102]

Kaempferitrin (**119**), which was isolated from the butanol fraction of *Bauhinia forficata* Link (Fabaceae) leaves, was found to decrease serum glucose levels significantly both in normal and alloxan-induced diabetic rats. The effect was maintained throughout the period studied. However, kaempferitrin (**119**) failed to change the glucose tolerance curve during the time course.[99]

Water decoctions of the plant *Cecropia obtusifolia* Bertol. (Cecropiaceae), also called “Guarumbo” in Mexico, are traditionally used for the treatment of Type 2 diabetes. Bioassay-guided isolation of the active components from this plant, led to the identification of isoorientin (**134**), a natural flavonoid. Further pharmacological tests showed that isoorientin (**134**) exhibited a significant hypoglycemic effect comparable to that of glibenclamide, an antidiabetic drug stimulating insulin release in STZ-induced diabetic rats.[110]

The flavone eupatilin (**111**), isolated from *Artemisia princeps* Pampan. (Asteraceae), was used to study the dose-response effects on blood glucose regulation and pancreatic β-cell function in C57BL/KsJ-db/db diabetic mice, a model for diabetic dyslipidemia. It was demonstrated that the supplementation of eupatilin (**111**) significantly lowered the fasting blood glucose concentration, glycated hemoglobin and plasma glucagon levels, while it simultaneously increased the hepatic glycogen content, plasma insulin and adiponectin levels.[62]

Genistein (**112**) and daidzein (**113**), the major isoflavones of soybean (*Glycine max*) (Fabaceae), were found to lower blood glucose, increase the insulin/glucagon ratio and the C-peptide level and to preserve β-cells of the pancreas in the non-obese diabetic mice, a model of the onset of Type 1 diabetes. Improved plasma TG and free fatty acid concentrations were also observed with the supplements of genistein (**112**) and daidzein (**113**). These results suggest the beneficial effect of genistein (**112**) and daidzein (**113**) on diabetes.[111]

Another study showed that the supplement of genistein (**112**) increased the plasma insulin level and glucokinase level while decreased the HbA1c and glucose-6-phosphatase level of the STZ-induced diabetic rats. An increase of hepatic superoxide dismutase, catalase and glutathione peroxidase activities were also observed in the groups treated with genistein (**112**). The results again suggested genistein's beneficial potential for hyperglycemia and related diabetic complications.[79]

In an investigation of a natural flavonoid, apigenin 6-C-(2"-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**135**), which was isolated from *Averrhoa carambola* L. (Oxalidaceae) leaves, an acute lowering effect on blood glucose was observed in hyperglycemic rats. The compound could also significantly stimulate the ^{14}C-glucose uptake and the glucose-induced insulin secretion[112]. Apigenin 6-C-β-L-fucopyranoside (**136**), another natural flavonoid with the same aglycone (**109**) from this plant, showed a similar effect in hyperglycemic normal rats.[113]

Naringenin (**137**), which is a citrus-derived (Rutaceae) flavanone, displayed both lipid-lowering and insulin-like properties. In low-density lipoprotein (LDL) receptor-null mice fed with a western diet, a model of diet-induced insulin resistance, the addition of naringenin (**137**) could decrease plasma lipids, liver TG and cholesterol, normalize blood glucose, plasma insulin, and glucose tolerance. These effects prove naringenin's potential on metabolic syndrome.[114]

Naringin (**129**), a naringenin (**137**) glycoside, and hesperidin (**128**), also citrus-derived bioflavanones, both significantly decreased blood glucose, elevated hepatic glucokinase activity and glycogen concentration in C57BL/KsJ-db/db mice. Two bioflavonoid-supplemented groups of mice also demonstrated significantly increased plasma insulin, C-peptide, and leptin levels. The results suggest that hesperidin and naringin help to prevent the progression of hyperglycemia.[81]

Besides, hesperidin (**128**) also proved to have a preventive action against diabetic embryopathies. With a decrease on maternal glycemia, hesperidin (**128**) treatment increased the number of implantations, foetuses and the foetal weights. A decrease of maternal body weight in pregnant diabetic mice was observed as well. In addition, the malformations and resorption rates were reduced.[115]

Phloridzin (**138**), a dihydrochalcone, is naturally occurring in apples, species *Malus domestica* (Rosaceae). Diets containing phloridzin (**138**) showed antihyperglycemia activities, however, unlike quercetin (**35**), it failed to improve hypoinsulinemia and tissue lipid peroxidation in STZ-induced diabetic mice. In a gene expression responses test, dietary phloridzin (**138**) indicated to reverse the abnormal overexpression of sodium/glucose cotransporter 1, cytochrome P450 2B10, and epoxide hydrolase 1 in the small intestine, as well as that of glucose transporter 2 in the kidney of STZ-induced diabetic mice.[116]

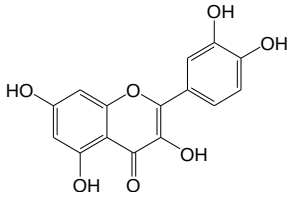
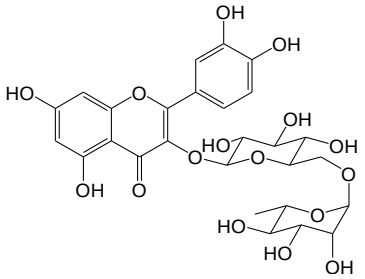
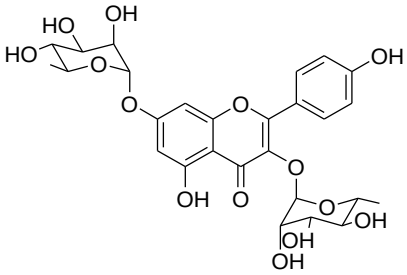
Two chalcones, 4-hydroxyderricin (**139**) and xanthoangelol (**140**) were isolated from the ethanol extract of *Angelica keiskei* (Apiaceae), a Japanese herb named "Ashitaba". Two weeks feeding of basal diets containing 4-hydroxyderricin (**139**) and xanthoangelol (**140**) both decreased blood glucose levels of KK-Ay mice, an animal model of metabolic syndrome. In a glucose uptake assay, 4-hydroxyderricin (**139**) showed stronger activity, compared with xanthoangelol (**140**)**.**[117]

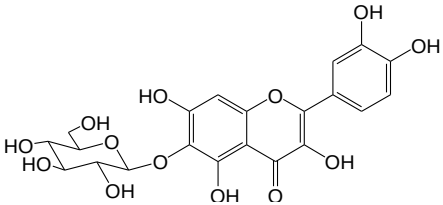
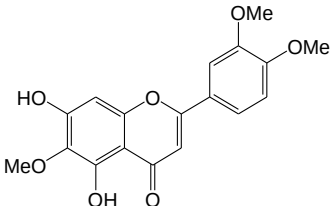
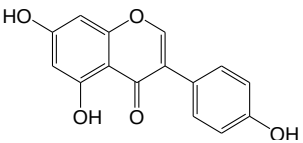
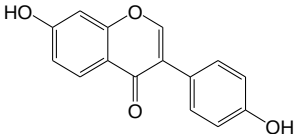
Green tea, the leaves of *Camellia sinensis* (Theaceae), is a significant source of a type of flavonoids called catechins. The green tea catechins include (-)-epigallocatechin gallate (EGCG, **141**), (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epicatechin (**142**). An injection of EGCG (**141**) produced a significant decrease in serum glucose levels, food intake and body weight in Sprague Dawley rats, as well as in lean and obese Zucker rats.[118] Additionally, (-)-epicatechin (**142**), the active compound in the extract of *Pterocarpus marsupium* Roxb bark, which is traditionally used in Indian folk medicine to treat diabetes, also showed significant hypoglycemic effects.[119]

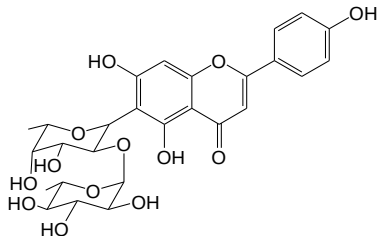
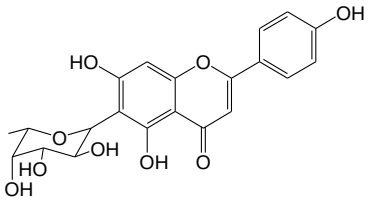
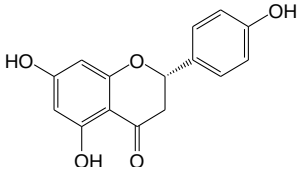
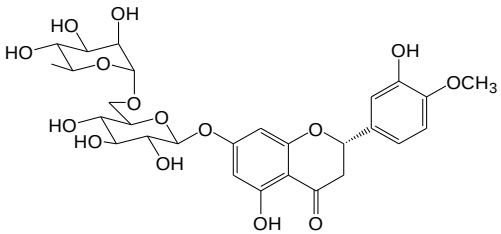
Anthocyanins, with their aglycone derivatives, called anthocyanidins, belong to the flavonoids. Of these groups, pelargonidin (**143**), which is abundantly present in common bean (*Phaseolus vulgaris* L.) (Fabaceae), together with pelargonodin 3-O-rhamnoside and pelargonidin 3-galactoside, displayed significant hypoglycemic effects in diabetic rats. Moreover, pelargonidin (**143**) treatment was found to decrease blood glucose, normalize glucose tolerance and improve serum insulin levels in STZ-induced diabetic rats. A decrease of serum superoxide dismutase and catalase levels, with an increase of malondialdehyde level, was observed in the experiment as well.[120]

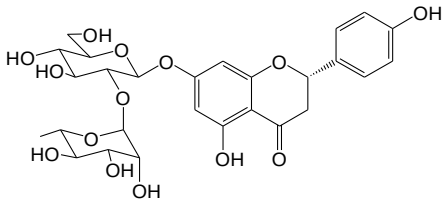
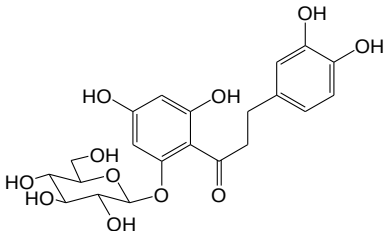
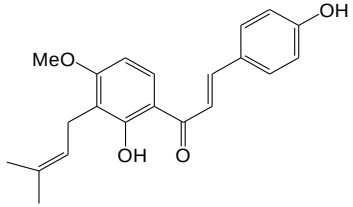
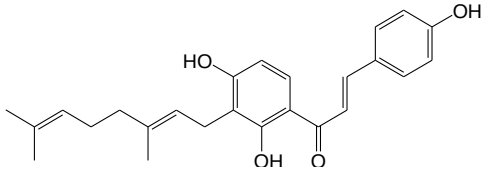
Silybinin (**144**) is the major flavonolignan from the extracts of milk thistle seed, *Silybum marianum* (Asteraceae). In patients with Type 2 diabetes mellitus and associated chronic liver disease, a better glucose and lipid metabolism was observed after the treatment with a silybinin β-cyclodextrin complex agent. The same trend towards normalization was achieved in mean daily blood glucose, HbA1c and insulin levels. Moreover, insulin sensitivity, which was estimated by MDA, an index of oxidative stress, was improved after the use of silybinin (**144**) in Type 2 diabetes patients.[121]

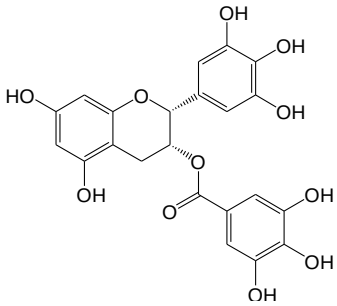
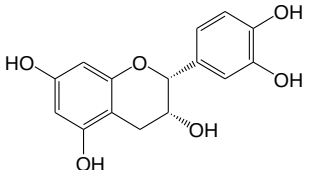
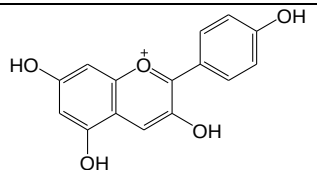
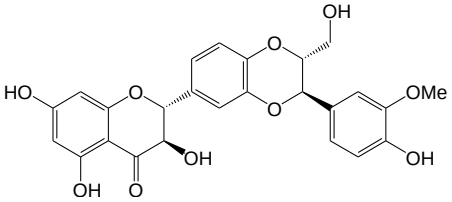
Table 1.4 Natural flavonoids with antidiabetic activities reported from in vivo studies

Flavonoid	Category	Struture	Dosage	Marker/parameter tested/evaluated	Literature reference
Quercetin (**35**)	flavonols	 **35**	10, 15 mg/kg	decreased the plasma glucose, cholesterol and TG, normalized the glucose tolerance; decreased serum glucose, MDA and NO, elevated serum insulin and antioxidant enzyme activities; decreased serum glucose and MDA, restored vascular function, increased antioxidant capacity	[56]; [57]; [58]
Rutin (**38**)	flavonol glycosides	 **38**	25, 50, 100 mg/kg	decreased fasting plasma glucose, HbA1c, insulin, C-peptide, hemoglobin, protein levels, TBARS and lipid hydroperoxides, increased non-enzymatic antioxidants	[102]
Kaempferitrin (**119**)	flavonol glycosides	 **119**	50, 100, 200 mg/kg	decreased blood glucose	[99]

Isoorientin (**134**)	flavonol glycosides	 **134**	3 mg/kg	decreased plasma glucose	[110]
Eupatilin (**111**)	flavones	 **111**	50, 200 mg/kg	lowered fasting blood glucose, HbA1c and plasma glucagon, increased glycogen content, plasma insulin and adiponectin	[62]
Genistein (**112**)	isoflavones	 **112**	200 mg/kg 600 mg/kg	decreased blood glucose level, increased the insulin/glucagon ratio and the C-peptide, improved plasma TG and free fatty acid; increased the plasma insulin and glucokinase, decreased HbA1c and glucose-6-phosphatase	[111]; [79]
Daidzein (**113**)	isoflavones	 **113**	200 mg/kg	decreased blood glucose level, increased the insulin/glucagon ratio and the C-peptide, improved plasma TG and free fatty acid	[111]

Apigenin 6-C-(2"-O-α-L-rhamnopyranosyl)-β-L-fucopyranoside (**135**)	flavone C-glycosides	 **135**	50 mg/kg	lowered blood glucose level, stimulated glucose-induced insulin secretion	[112]
Apigenin 6-C-β-L-fucopyranoside (**136**)	flavone C-glycosides	 **136**	50 mg/kg	lowered blood glucose level, stimulated glucose-induced insulin secretion, regulated glycogen synthesis	[113]
Naringenin (**137**)	flavanones	 **137**	3% w/w	decreased plasma lipids, liver TG and cholesterol, normalized blood glucose, plasma insulin and glucose tolerance	[114]
Hesperidin (**128**)	flavanone glycosides	 **128**	200 mg/kg 20 mg/kg	reduced blood glucose, elevated hepatic glucokinase activity and glycogen concentration, increased plasma insulin, C-peptide and leptin levels; decreased maternal body weight, glycaemia, malformations and resorption rates, enhanced implantations, foetuses and foetal weights	[81]; [115]

Naringin (**129**)	flavanone glycosides	 **129**	200 mg/kg	reduced blood glucose, elevated hepatic glucokinase activity and glycogen concentration, increased plasma insulin, C-peptide and leptin levels	[81]
Phloridzin (**138**)	chalcones	 **138**	0.5 % diet	improved hyperglycemia, reversed the abnormal overexpression	[116]
4-Hydroxyderricin (**139**)	chalcones	 **139**	0.15 % diet	reduced blood glucose	[117]
Xanthoangelol (**140**)	chalcones	 **140**	0.15 % diet	reduced blood glucose	[117]

(–)-Epigallocate-chingallate (**141**)	catechins	 **141**	82 mg/kg	decreased glucose serum	[118]
(–)-Epicatechin (**142**)	catechins	 **142**	15, 30 mg/kg	decreased glucose serum	[119]
Pelargonidin (**143**)	anthocyanins	 **143**	3 mg/kg	decreased blood glucose levels, improved serum insulin and glucose tolerance, increased superoxide dismutase and catalase, lowered MDA and fructosamine	[120]
Silybinin (**144**)	flavonolignan	 **144**	135 mg/day	decreased fasting blood glucose	[121]

Abbreviations: TG, triglyceride; MDA, malondialdehyde; NO, nitric oxide; HbA1c, glycated haemoglobin; TBARS, thiobarbituric acid reactive substances

1.3.4 Studies on flavonoids used for the treatment of diabetic complications

Diabetes is a chronic, life-long disease which needs careful management. It can lead to various complications such as cardiovascular disease, kidney failure, nerve damage and blindness. The beneficial effects of flavonoids are numerous, involved in battling many types of diabetic complications. Their specific bioactivities on different diabetic complications, with the parameters tested, are stated in Table 1.4.

1.3.4.1 Treatment of diabetes-related cardiovascular disease

Diabetes has become a major risk factor for cardiovascular disease (CVD), and in turn, the CVD is the main cause of death in people with diabetes.[122] More and more epidemiological studies have suggested an inverse association between the dietary intake of flavonoids and the risk for vascular disease, which attracted scientists' interest on flavonoids.[123]

Pycnogenol, a flavonoid-rich (catechin, epicatechin, taxifolin, and procyanidins) extract from the bark of the French maritime pine (*Pinus maritima*) (Pinaceae), was reported to show beneficial effects on the cardiovascular system. Pycnogenol treatment in Type 2 diabetes individuals achieved blood pressure control, decreased serum endothelin-1, an endothelial-derived vasoconstrictor peptide associated with modulating the vascular tone. Additionally, pycnogenol mediated a significant improvement in serum LDL cholesterol, a predictor of cardiovascular disease in individuals with diabetes. The results suggested that pycnogenol has beneficial effects in Type 2 diabetes-related cardiovascular disease.[124]

For those drugs with poor solubility, cyclodextrins can be used in pharmaceutical preparations to increase the bioavailability. In a study of the effects on left ventricle dysfunction in STZ-induced diabetic rats, β-cyclodextrin complexes of quercetin (**35**) and rutin (**38**) significantly decreased serum TG and cholesterol levels. In the histopathological studies, the degree of myocardial necrosis was less in rutin-treated animals, with decreased myocardial fructose levels. This investigation demonstrates that quercetin (**35**) and rutin (**38**) have a cardioprotective activity.[125]

In another study, the treatment with phytoestrogenic isoflavones genistein (**112**) and daidzein (**113**) effectively improved glucose toxicity-induced cardiac mechanical malfunction in ventricular myocytes.[126] Both results suggest that the flavonoids-related preparations have a therapeutic role against diabetes-associated cardiac defects.

1.3.4.2 Treatment of diabetic nephropathy

Diabetic nephropathy is one of the most serious complications in diabetes mellitus, and it could cause end-stage renal failure among patients undergoing chronic hemodialysis therapy[127]. Together with a good hypoglycemic activity, flavonoids could be used to prevent the development and progression of nephropathy.

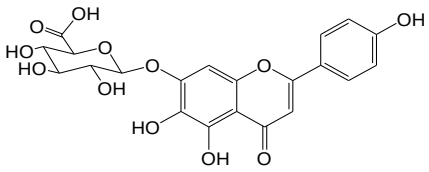
The flavonoid mixture silymarin, was tested for its effect on oxidative stress and renal

tissue morphology in alloxan-induced diabetic rats. Tissue damage caused by alloxan was prevented by silymarin treatment and the activity and gene expression of the three antioxidant enzymes (SOD, glutathione peroxidase and catalase) were restored after the treatment with silymarin. These results suggest that silymarin can be useful in the treatment of diabetic nephropathy.[128]

Supplementation of green tea catechin, recovered the destroyed balance between prostacyclin and thromboxane A2, an index of kidney function, in the kidney microsomes of STZ-induced diabetic rats. Catechin also adjusted the kidney thiobarbituric acid reactive substance (TBARS) content, an index of lipid peroxidation, to the normal level.[129,130] Besides, treatment with tea catechin significantly reduced albuminuria in diabetic rats. Moreover, it helped to normalize the glomerular hypertrophy and interstitial fibrosis.[131] These results suggest that tea catechin can contribute to the prevention of diabetic kidney failure.

In a study of EGCG (**141**), a type of catechins abundant in green tea, treated rats with subtotal nephrectomy and STZ injection showed decreased hyperglycemia, suppressed proteinuria and lipid peroxidation. In a renal damage pathological test, reduced renal advanced glycation endproduct accumulation and related protein expression in the kidney cortex were observed. These results proved EGCG's beneficial effect on abnormal glucose metabolism-associated renal damage.[132]

Another study showed that breviscapine (**145**), a flavonoid isolated from the Chinese herb *Erigeron breviscapus*, significantly alleviated albuminuria, glomerular hypertrophy and tubulointerstitial injury in STZ-induced diabetic rats, which involves a renoprotective effect through suppression of renal macrophage recruitment.[133]



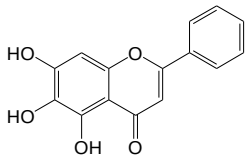
Breviscapine **145**

1.3.4.3 Treatment of oxidative stress-associated diabetic complications

Diabetes is closely associated with oxidative stress, by which it produces excessive free radicals to contribute to the development of diabetes, and further to accelerate diabetic complications.[134] Natural flavonoids are helpful in many oxidative stress problem related diabetic complications such as diabetic neuropathic pain, renal and liver damage.

Several laboratory studies showed that quercetin (**35**) may be beneficial in diabetic neuropathy. Quercetin-treated STZ-induced diabetic and nondiabetic mice both exhibited a marked increase in tail-flick latencies.[135] Additionally, chronic treatment with quercetin (**35**) significantly produced an improvement of cold allodynia and thermal hyperalgesia in STZ-induced diabetic rats.[136] In a study of the evaluation of the flavonoid

baicalein (**146**) on diabetic peripheral neuropathy, this compound was shown to reverse nerve conduction deficit and small sensory nerve fiber dysfunction.[137]



Baicalein **146**

Another report showed that quercetin (**35**) treatment significantly decreased oxidative stress with markers such as superoxide dismutase, catalase, serum creatinine and blood urea nitrogen levels increased in STZ-injected rats. Moreover, renal dysfunction evidence such as proteinuria, polyuria, creatinine and urea clearance were improved.[138] The treatment with rutin (**38**) adjusted the antioxidant status of diabetic rats by decreasing lipid peroxidative products and by increasing enzymatic (SOD, catalase, glutathione peroxidase and glutathione reductase) and nonenzymatic antioxidants (reduced glutathione, vitamin C and E).[101] The findings suggest that the protective role of flavonoids on diabetic organs damage, probably is materialized through their antioxidative action.

1.3.4.4 Treatment of diabetes-related hyperlipidemia

Diabetes mellitus is a metabolic syndrome, with hyperglycemia and hyperlipidemia often seen together in this disease. Flavonoids are found to have not only antidiabetic activity but also a hypolipidemia effect.

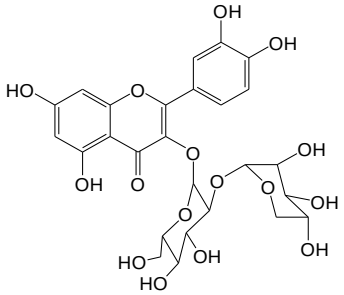
Rutin (**38**) was found to have a beneficial effect on lipids, lipoproteins, lipid-metabolizing enzymes and glycoproteins.[139] Apigenin (**109**) was demonstrated to alleviate hyperlipidemia and atherosclerosis in diabetes, by increasing phosphorylation of hepatocellular adenosine monophosphate-activated protein kinase (AMPK), which plays an important role in the pathogenesis of hyperlipidemia in diabetes.[140]

1.3.4.5 Treatment of diabetic complications associated with advanced glycation end-products

Advanced glycation end-products (AGEs) are a diverse class of biomolecules resulting from non-enzymatic glycation of amino acids, peptides, proteins or amines with reducing sugars, such as glucose and fructose, or its autooxidation products. They are specifically the result of the Maillard reaction.[103] The AGEs play an important role in the development of diabetic complications, such as neuropathy, retinopathy and nephropathy.[107] Flavonoids have the proven ability to inhibit the formation of AGEs.

In a study of the protective potential against protein damage of four flavonoids, isoquercitin (**36**) and hyperin (**115**) exerted a stronger inhibitory activity against the formation of AGEs in vitro. The activities were more potent than that of aminoguanidine, a known glycation inhibitor.[105] In another study, isoquercitin (**36**) with two other flavonol

glycosides, quercetin 3-O-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside (**147**) and astragalin (**41**), isolated from the leaves of *Eucommia ulmoides* Oliv. (Eucommiaceae), were demonstrated as glycation inhibitors as well.[141]



Quercetin 3-O-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside **147**

1.3.4.6 Treatment of other diabetic complications

Poorly controlled diabetics can cause an accelerated development of the following complications.

Diabetic retinopathy (damage to the retina) is also a type of complication from diabetes mellitus, which induces blindness at the end. Treatment with genistein (**112**) significantly inhibited retinal vascular leakage in STZ-induced diabetic rats. A decrease of retinal albumin, phosphotyrosine levels and proliferating cell nuclear antigen were observed as well.[142]

Diabetes also causes depression, along with the inefficient plasma glucose control and increased risk of diabetic complications. Quercetin (**35**) showed a potential in therapy for depression associated with diabetes. It markedly reduced the immobility period in STZ-induced diabetic mice, compared with that of fluoxetine and imipramine, the classical antidepressants.[143]

Osteopenia (lowered bone mineral density) is also a diabetic complication. Myricetin (**108**) displayed protective effects on 2-deoxy-D-ribose-induced cytotoxicity in osteoblastic MC3T3-E1 cells and osteoblast dysfunction.[144] A similar activity was found after treatment with kaempferol (**40**).[145] These results suggest that both myricetin (**108**) and kaempferol (**40**) are beneficial in diabetes-related bone diseases.

Table 1.4 Effects of flavonoids in the treatment of diabetic complications

Flavonoid	Diabetic complication	Marker/parameter tested/evaluated	Literature reference
(–)-Epigallocatechin-3-O-gallate (**141**)	diabetic nephropathy	reduction of kidney weight, TC, TBARS, serum urea nitrogen, serum creatinine, creatinine clearance and urinary protein excretion	[132]
Apigenin (**109**)	hyperlipidemia	increase of AMPK and ACC phosphorylation, prevention of high-glucose-induced lipid accumulation	[140]
Breviscapine (**145**)	diabetic nephropathy	reduction of kidney weight, urinary albumin excretion rate, tubulointerstitial injury, MDA, increase of SOD, CAT and GSH-PX	[133]
Catechins	diabetic nephropathy	improvement of prostacyclin / thromboxane A2 ratio, decrease of TBARS; reduction of urinary albumin excretion rate, normalization of interstitial fibrosis area	[129, 130]; [131]
Daidzein (**113**)	cardiovascular disease	reduction of mechanical malfunction in ventricular myocytes	[126]
Genistein (**112**)	cardiovascular disease diabetic retinopathy	reduction of mechanical malfunction in ventricular myocytes; decrease of retinal vascular permeability, retinal albumin, phosphotyrosine and proliferating cell nuclear antigen	[126]; [142]
Isoquercitin (**36**), hyperin (**115**)	AGE formation	inhibition of glycation	[105]
Myricetin (**108**), kaempferol (**40**)	osteopenia	increase of cell survival, ALP activity, collagen, osteocalcin, osteoprotegerin and calcium deposition; decrease of MDA, protein carbonyl and advanced oxidation protein products	[144]; [145]
Pycnogenol	cardiovascular disease kidney failure	decrease of endothelin-1, LDL-cholesterol and urinary albumin	[124]

Quercetin (**35**)	cardiovascular disease	decrease of body mass, TG, cholesterol, myocardial fructose;	[125];
	diabetic nephropathy	decrease of proteinuria, polyuria, serum creatinine, blood urea nitrogen and MDA, increase of creatinine and urea clearance, SOD, CAT, GSH-PX;	[138];
	diabetic neuropathy	increase of tail withdrawal latency;	[135];
		attenuation of cold allodynia, hyperalgesia, increase of tail immersion and hot-plate pain threshold;	[136];
	AGE formation	inhibition of glycation;	[108];
	depression	reduction in immobility period	[143]
Quercetin 3-O-α-L-arabinopyranosyl-(1→2)-β-D-glucopyranoside (**147**), astragalin (**41**), isoquercitin (**36**)	AGE formation	inhibition of glycation	[141]
Rutin (**38**)	Hyperlipidemia	decrease of cholesterol, TG, free fatty acids, phospholipids, LDL-cholesterol, very LDL-cholesterol, HMG-CoA reductase, glycoproteins, TBARS and HP increase of LPL, LCAT, HDL-cholesterol;	[139]
	oxidative stress-related diseases	increase of GSH, Vitamin C, Vitamin E, SOD, CAT and GSH-PX	[101]
Silymarin	diabetic nephropathy	restoration of SOD, GSH, CAT	[128]

Abbreviations: TC, total cholesterol; TBARS, thiobarbituric acid reactive substances; AMPK, AMP- activated protein kinase; ACC, acetyl-CoA carboxylase; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase; ALP, alkaline phosphatase; LDL, low-density lipoprotein; TG, triglyceride; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LPL, lipoprotein lipase; LCAT, lecithin-cholesterol acyltransferase; HDL, high-density lipoprotein; HP, hydroperoxides; GSH, glutathione

1.3.5 Conclusion and further perspectives

As the incidence of diabetic patients around the world continues to rise and due to the complexity and diversity of the disease etiology, together with its multiple complications, medicinal researchers have increasing responsibilities to seek for effective drugs for the treatment of diabetes mellitus. Natural products and their derivatives have been among the most productive source of bioactive molecules in drug discovery.[146] The contribution of natural products to the development of antidiabetic agents is clearly evident, as we discussed at the beginning. Flavonoids are a large group of natural products, and according to the literature, more than 10,000 different flavonoids from widespread plant sources have been reported with each year, hundreds of newly identified flavonoids being recorded.[147]

Flavonoids exhibit multiple biological activities, such as antiviral, antiinflammatory, antiproliferative and anticancer activity, attracting the interest of scientists. Their well noted ability to reduce free radical formation and to scavenge free radicals highlights the antioxidant activity of flavonoids.[148] Recent investigations have demonstrated that flavonoids are also very promising antidiabetic agents. The major potential benefits of natural flavonoids discussed in this overview clearly demonstrate that these substances represent remarkable lead compounds with a diversity of molecular structure and biological activity, in relation to the antidiabetic drug discovery process. Although considerable scientific progress has been made in unraveling the effect of flavonoids on diabetes, diabetic complications and their mechanism of action, especially over the past 10 years, the pharmacokinetic factors including the absorption, metabolism and toxicological aspects of flavonoids, together with the missing steps in the flavonoid-signaling network based on the complex mechanism of insulin action shall have to be concerned.[149]

On the other hand, a large number of plant extracts have gained gratifying results in experimental animal models and clinical studies for the treatment of diabetes mellitus. Unlike a single chemical entity aimed at a specific single target, multi-flavonoid-rich plant extracts managed to alleviate the disorder of diabetes mellitus, probably through an integrated effect upon multi-target sites. Flavonoids are very common in several comestible fruits and vegetables as parts of our daily food consumption.[150] There is no doubt that plant extracts should be viewed as a potential complementary treatment for diabetes.

Diabetes mellitus is a chronic disease which is difficult to cure. Without the control of blood glucose it fastly develops diabetic complications. The evidences from published literature demonstrate that some flavonoids, such as quercetin (**35**) and rutin (**38**), show multifunctional health benefits in diabetes and its complications due to their acting on many targets with high affinity. Nowadays, more antidiabetic research studies are focused on its pathogenesis, and the studies of the action of active ingredients are relatively small. Flavonoids, as an important class of natural products with high pharmacological activities, provide new insights into the potential role of lead

compounds in diabetes treatment. The knowledge offered from this overview should help to provide leads to the ultimate goal of developing new more efficient therapeutic drugs for the treatment of diabetes mellitus and its complications, in spite of the fact that more clinical and experimental studies are obviously needed.

1.4 Purpose and the goal of the present research

Diabetes mellitus, as a worldwide chronic disease, is still rapidly increasing. Diabetes and its complications have caused serious burden on world's economy. Most notably, more than 80% of diabetes deaths occur in low and middle-income countries.

The traditional Chinese medicine, which uses natural medicines originating mainly from herbs by distinctive traditional medical theory, has demonstrated valuable practice and shows a bright future in the therapy of diabetes and its complications. Many prescriptions including Simple Recipes and Compound Recipes have been applied in the therapy of diabetes with a varying medical emphasis on different symptoms of the disease.[52] Compared to the classical drugs (insulin and oral hypoglycemic agents) for the treatment of diabetes, some folk secret recipes showed outstanding curative effects with fewer side effects and lower cost for the maintenance of treatment of this lifelong disease. In this respect, the rich Chinese traditional medicines covering large numbers of medicinal plants offer a great potential for such discoveries. It is important to elucidate the active principles and to understand the mechanism of action of the natural medicines from traditional Chinese medicinal plants used in the treatment of diabetes.

The genus *Gynura* Cass. (Asteraceae family) displays a broad range of uses in traditional Chinese medicine. As local secret recipes, *G. bicolor* and *G. divaricata* have been used to treat diabetes in Jiangsu, Zhejiang and Sichuan province in south China and showed excellent effects. Certain patients can successfully control their blood glucose levels in a normal status by eating the leaves of these two species or by drinking them as a tea.[151] In China, *G. divaricata* is mostly a wild species, while *G. bicolor* is cultivated as a vegetable in some regions. These two plants have been introduced and cultivated in the Nanjing Botanical Garden (Mem. Sun Yat-Sen) since 2000 by the Jiangsu Provincial Key LAB For Plant EX SITU Conservation.

However, literature reports on these plants are limited and little is known about the antidiabetic chemical constituents from the plants. Our research group in China performed pharmacological tests to prove the hypoglycemic effect of these two plants. These tests showed that both ethyl acetate and butanol extracts of *G. bicolor* showed a significant effect on lowering the blood glucose level in normal and alloxan-diabetic mice, while the ethyl acetate and butanol extracts of *G. divaricata* were effective on lowering the blood glucose level in alloxan-diabetic mice in a low dosage. These results indicate that *G. bicolor* and *G. divaricata* are promising plants for the development of natural products for the treatment of diabetes mellitus. Therefore, it is very important to perform further research on the isolation of the active chemical constituents and on their mechanism of action.[151]

Based on the above consideration, the present research was undertaken to investigate the chemical ingredients of unstudied *G. bicolor* and *G. divaricata*, and to study their hypoglycemic bioactivity by evaluation of the pharmacological effects in *in vitro* models. Combined with the chemical study and biotesting of the hypoglycemic compounds of these two plants, the final goal of these research activities is to obtain some lead medicinal compounds for the treatment of diabetes mellitus, although this may, of course, not be realized within the scope of this thesis.

Furthermore, essential oils are volatile secondary metabolites produced by aromatic plants and play an important role in the protection and life cycle of plants.[152] Due to their interesting biological effects, they play an important role in traditional pharmacopoeia. Therefore, for the first time, the volatiles from fresh leaves of *G. bicolor* and *Gynura divaricata* are studied using a SPME method coupled with GC-MS.

Chapter 2

STUDY OF THE CHEMICAL CONSTITUENTS OF THE AERIAL PARTS OF *GYNURA BICOLOR*

2.1 Introduction

Gynura bicolor (Roxb. & Willd.) DC is a herb, about 50-100 cm high; the stems are glabrous, rather woody at the base; leaves are obovate or oblanceolate; blade adaxially green while abaxially becoming purplish when dry (Figure 2.1). Capitula are numerous, peduncles about 3-4 cm; involucres campanulate; florets are orange to reddish; corolla distinctly exceeding involucres, 13-15 mm; tube 10-12 mm; lobes are ovate-triangular. Anthers are rounded at the base, or slightly acute. Style branch tips subulate, papillose. Achenes are brownish, cylindric, glabrous, 10-15-ribbed. Pappus white, silky, easily deciduous. The flower time is May to October.[12]

Figure 2.1. *Gynura bicolor* (Roxb. & Willd.) DC

Gynura bicolor, which has been cultivated as a popular vegetable, is not only known to be nutritive but is also used for the treatment of diabetes in the south of China. Our previous pharmacological tests proved that the ethyl acetate and *n*-butanol extracts of the aerial parts of the plant had significant effects on lowering blood glucose level in normal and alloxan diabetic mice.[151] However, there are few reported phytochemical investigations of this species. To our knowledge, only two studies investigated the volatiles[153,154] and one research reported on some anthocyanins[155] from this plant. Therefore, this further research was conducted on the isolation and identification of the active chemical constituents from *G. bicolor*. The bioassay study of the isolated compounds on potential hypoglycemic activity, en route towards the development of natural products for the treatment of diabetes mellitus, was planned as well.

2.2 Experimental

2.2.1 Plant materials

The aerial parts of *G. bicolor* for chemical constituent investigation were collected in June 2010 in Nanjing Botanical Garden Mem. Sun Yat-sen, in the south of the Zijin Mountain, Nanjing, China. The plant was identified by Professor Guo Rong-lin at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. A voucher specimen (No. 510310-1) was deposited in the herbarium, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences.

2.2.2 Instrumentation

Silica gel 60 (0.015-0.040 mm for column chromatography; Merck) was used as normal phase, whereas LiChroprep RP-18 (40-63 μm particle size; Merck) was used as reversed phase column material. MCI gel CHP20P (75-150μm, 400-600 Å mean pore size; Mitsubishi Chemical Corp.) and Sephadex LH-20 (GE healthcare) were used for column chromatography as well. TLC analyses were carried out on silica gel plates (KG60-F254, Merck). The melting point was measured with an X-6 micro-melting point apparatus (Beijing Tech). ^{1}H and ^{13}C NMR spectra were obtained on a JEOL 300 spectrometer. The HPLC instrument consisted of an Agilent (Waldbronn, Germany) model 1100 liquid chromatograph with diode array detector. The HPLC-MS consisted of Agilent LC/MSD 1100 series, and the prep-LC consisted of Agilent 1100 serious equipped with VWD detector and automatic fraction collector. Automatic flash chromatography was performed by The Reveleris Flash System from GRACE, United States.

2.2.3 Extraction and Purification

2.2.3.1 Extraction

Dry aerial parts of *G. bicolor* (2.9 kg) were cut into small pieces and extracted with 80% aqueous ethanol at 70 ˚C twice to afford 295 g of crude extract after evaporation in vacuo of the solvent. The crude extract was dissolved in ethanol, coated on silica and dried, then successively extracted exhaustively with *n*-hexane, CH_2Cl_2, EtOAc and MeOH. The solvent was removed and the sample was dried before starting the next extraction with a new solvent. Extraction was executed with stirring at 40 ˚C of water-bath heating. The extracts were filtered and concentrated by evaporation under reduced pressure with a rotavapor at 40 ˚C to afford a dark green *n*-hexane residue (0.8 g, 0.03% yield), a dark CH_2Cl_2 residue (3.4 g, 0.12% yield), a dark EtOAc residue (7.7 g, 0.27% yield), and a dark brown MeOH residue (121.3 g, 4.18% yield).

2.2.3.2 Study of the dichloromethane extract of the aerial parts of *G. bicolor*

The CH_2Cl_2 extracted fraction (3.4 g) was adsorbed on 8.0 g of silica gel and further fractionated by column chromatography over silica gel, eluted with CH_2Cl_2 containing increasing concentrations of MeOH (Table 2.1). The fractions were collected in 50 mL tubes and after monitoring by TLC, a total of 6 different combined fractions (Fr. B1-6) were obtained (Table 2.2).

Table 2.1 Programme for fractionation of the CH_2Cl_2 extract of *G. bicolor* by column chromatography

Mobile phase	Gradient elution		Solvent volume (mL)
	Start	End	
CH_2Cl_2	100	100	1000
CH_2Cl_2/MeOH	100:2	100:2	1020
CH_2Cl_2/MeOH	100:4	100:4	1040
CH_2Cl_2/MeOH	100:10	100:10	1430
CH_2Cl_2/MeOH	100:25	100:25	500
CH_2Cl_2/MeOH	100:100	100:100	400
MeOH (clean up)	100	100	300

Table 2.2 Column chromatography fractionation of the CH_2Cl_2 extract of *G. bicolor*

Fraction	Weight (mg)
Fr. B1	1050
Fr. B2	600
Fr. B3	655
Fr. B4	280
Fr. B5	214
Fr. B6	302

2.2.3.2.1 Isolation of 5-(hydroxymethyl)furfural **148**

Fr. B1 (1050 mg) was adsorbed on a reverse phase LiChroprep RP-18 (1500 mg) and rechromatographed over a reverse phase column. After eluting by a gradient of water and methanol (50-100%) (Table 2.3) and monitoring by normal phase TLC, four combined fractions (Fr. B1-1~4) were obtained (Table 2.4).

Table 2.3 Programme for separation of fraction Fr. B1 of *G. bicolor* by column chromatography

Mobile phase	Gradient elution		Solvent volume (mL)
	Start	End	
MeOH/H_2O	50:50	50:50	300
MeOH/H_2O	70:30	70:30	300
MeOH/H_2O	85:15	85:15	300
MeOH (clean up)	100	100	300

Table 2.4 Column chromatography fractionation of Fr. B1 of *G. bicolor*

Fraction	Weight (mg)
Fr. B1-1	176
Fr. B1-2	105
Fr. B1-3	144
Fr. B1-4	133

Fr. B1-2 (105 mg) was submitted to further purification with an automatic flash chromatography system on a normal phase column eluted by a gradient of CH_2Cl_2 and methanol (0-8%). The automatic flash chromatography conditions were (Table 2.5 and 2.6):

Table 2.5 Automatic flash chromatography conditions of Fr. B1-2 of *G. bicolor*

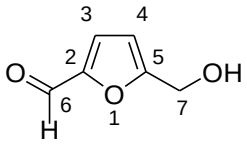
Run conditions	
Cartridge:	Reveleris 12 g Silica
Solvent A:	CH_2Cl_2
Solvent B:	Methanol
Flow rate:	30 mL/min
Detector:	ELSD

Table 2.6 Automatic flash chromatography gradient method of Fr. B1-2 of *G. bicolor*

Gradient Method		
Step	Time (min.)	%B
1	0	0
2	15	8

The eluates from 8.3-10.6 min were combined, concentrated by evaporation under reduced pressure with a rotavapor and finally dried on high vacuum to afford a pure compound (**148**, 7.3 mg) as a colorless oil.

By comparison of its ^{1}H, ^{13}C NMR and ESI-MS spectral data with those reported,[156] the compound **148** was identified as 5-(hydroxymethyl)furfural.



148

5-(Hydroxymethyl)furfural 148: colorless oil; ESI-MS m/z: 127 $[M+H]^+$; ^{1}H NMR δ ppm (DMSO-d_6, 300 MHz): 4.49 (2H, s, C$\underline{H}_2$OH), 6.59 (1H, d, *J* = 3.3 Hz, H-4), 7.48 (1H, d, *J* = 3.3 Hz, H-3), 9.53 (1H, s, C$\underline{H}$O); ^{13}C NMR δ ppm (DMSO-d_6, 75 MHz): 178.6 ($\underline{C}$HO), 162.8 (C-5), 152.3 (C-2), 125.0 (C-4), 110.3 (C-3), 56.5 ($\underline{C}H_2$OH).

2.2.3.2.2 Isolation of benzoic acid **78** and 4-hydroxybenzaldehyde **149**

Fr. B1-1 (176 mg) was subjected to column chromatography over MCI gel eluted by a gradient of water and methanol (20-100%) for further separation (Table 2.7).

Table 2.7 Programme for separation of fraction Fr. B1-1 of *G. bicolor* by column chromatography over MCI gel

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	150
MeOH/H_2O	40:60	200
MeOH/H_2O	60:40	200
MeOH/H_2O	80:20	150
MeOH (clean up)	100	100

Fractions MeOH/H_2O 40:60 and 60:40 (102 mg) were mixed together and re-separated with silica gel column chromatography using a gradient of CH_2Cl_2 and MeOH as eluent (Table 2.8). Based on their identity (monitoring by TLC under UV 254 and 365 nm), fractions which had similar chromatographic behaviour were combined for subsequent purification.

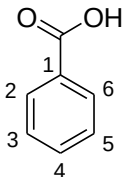
Table 2.8 Programme for separation of the fraction 40:60-60:40 of *G. bicolor*

Mobile phase	Gradient elution	Solvent volume (mL)
CH_2Cl_2	100	200
CH_2Cl_2/MeOH	100:1	600
CH_2Cl_2/MeOH	100:2	400
CH_2Cl_2/MeOH	100:10	300

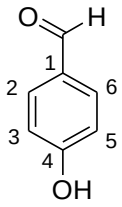
The CH_2Cl_2/MeOH 100:1-100:2 eluent was collected and two major bands with Rf value from 0.5-0.6 were observed on TLC plate (eluent: CH_2Cl_2/MeOH (10:1)) under UV detector. After solvent evaporation, a grey mixture was obtained. The mixture (28 mg) was dissolved in 1 mL HPLC grade methanol, then rechromatographed with an isocratic reversed phase preparative-HPLC elution with H_2O/MeOH (35:65) for further purification. Prep-HPLC conditions were: C18 column with guard catridge, 21.2 mm ID × 150 mm (5 μm); flow rate 6 mL/min; detection UV 254 nm; room temperature; injection volume 150 μL per time; running time 18 mins per injection; automatic fraction collector mode peak-based.

The eluates from 12.6-13.7 and 13.9-15.3 min were collected respectively, concentrated by evaporation under reduced pressure with a rotavapor and finally dried on high vacuum to afford two pure compounds **78** (5.4 mg) and **149** (6.7 mg) as white powder.

Analysis of their spectral data, together with comparison with data available in the literatures,[157,158] allowed to elucidate the structures of the compound **78** and **149** and to identify them as benzoic acid and 4-hydroxybenzaldehyde, respectively.



78



149

Benzoic acid 78: white powder; m.p. 118-119 °C; ESI-MS m/z: 121 $[M-H]^-$; ^{1}H NMR δ ppm ($CDCl_3$, 300 MHz): 7.46-7.52 (2H, m, H_{arom}), 7.60-7.65 (1H, m, H_{arom}), 8.12 (2H, d, *J* = 7.2 Hz, H_{arom}); ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 171.7 (COOH), 133.9 (C-4), 130.3 (C-2 and C-6), 129.3 (C-1), 128.6 (C-3 and C-5).

4-Hydroxybenzaldehyde 149: white powder; m.p. 108-109 °C; ESI-MS m/z: 121 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 6.89 (2H, d, *J* = 8.5 Hz, H-3 and H-5), 7.76 (2H, d, *J* = 8.5 Hz, H-2 and H-6), 9.74 (1H, s, CHO); ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 190.9 (CHO), 161.4 (C-4), 132.5 (C-2 and C-6), 130.1 (C-1), 116.0 (C-3 and C-5).

2.2.3.2.3 Isolation of vanillic acid **80**

Fr. B1-3 (144 mg) was subjected to column chromatography on MCI gel eluted by a

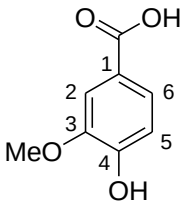
gradient of water and methanol (20-100%) for further separation (Table 2.9).

Table 2.9 Programme for separation of fraction Fr. B1-3 of *G. bicolor* by column chromatography over MCI gel

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	150
MeOH/H_2O	40:60	200
MeOH/H_2O	60:40	200
MeOH/H_2O	80:20	150
MeOH (clean up)	100	100

After monitoring by TLC with UV detection (254 and 365 nm), the 40% (methanol in water) eluent was collected for subsquent purification. Solvent removal by evaporation under reduced pressure yielded a grey mixture. The mixture (32 mg) was additionally purified by column chromatography on silica gel using CH_2Cl_2/MeOH (20:1) as eluent to afford a pure product (compound **80**, 3.1 mg).

By comparison of its ^1H, ^{13}C NMR and ESI-MS spectral data with those reported,[159] the compound **80** was identified as vanillic acid.



80

Vanillic acid 80: white powder; m.p. 210-211 °C; ESI-MS m/z: 167 $[M-H]^-$; ^1H NMR δ ppm (MeOH-d_4, 300 MHz): 3.90 (3H, s, OC<u>H</u>$_3$), 6.84 (1H, d, *J* = 8.3 Hz, H-5), 7.55 (1H, d, *J* = 1.8 Hz, H-2), 7.56 (1H, dd, *J* = 8.3, 1.8 Hz, H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 170.0 (<u>C</u>OOH), 152.7 (C-4), 148.7 (C-3), 125.3 (C-6), 123.0 (C-1), 115.8 (C-2), 113.8 (C-5), 56.4 (O<u>C</u>H$_3$).

2.2.3.2.4 Isolation of ficusic acid **150**

Fr. B2 (600 mg) was fractionated with an automatic flash chromatography system on a reversed-phase column (C-18, 12 g) eluted by a gradient of water and methanol (50-100%) to get four fractions (Fr. B2-1~4). The automatic flash chromatography conditions were (Table 2.10 and 2.11):

Table 2.10 Automatic flash chromatography conditions of Fr. B2 of *G. bicolor*

Run conditions	
Cartridge:	Reveleris 12 g C18
Solvent A:	Water
Solvent B:	Methanol
Flow rate:	30 mL/min
UV1 wavelength:	220 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 2.11 Automatic flash chromatography gradient method of Fr. B2 of *G. bicolor*

Gradient Method		
Step	Time (min.)	%B
1	0	50
2	20	60
3	20	80
4	20	100
5	2	100

The combined fractions were concentrated by evaporation under reduced pressure with a rotavapor and after monitoring by TLC with UV detection, four combined fractions (Fr. B2-1~4) were obtained (Table 2.12).

Table 2.12 Column chromatography fractionation of Fr. B2 of *G. bicolor*

Fraction	Weight (mg)
Fr. B2-1	248
Fr. B2-2	175
Fr. B2-3	64
Fr. B2-4	83

Fr. B2-1 (248 mg) was further subjected to column chromatography over MCI gel eluted by a gradient of water and methanol (20-100%) (Table 2.13). The 60% (methanol in water) eluent (56 mg) was collected for subsequent purification. Upon further silica gel column chromatography (CH_2Cl_2/MeOH, 100:1 to 10:1) (Table 2.14) and prep-HPLC (H_2O/CH_3CN, 75:25), a pure compound **150** (2.2 mg) was obtained. The prep-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID $\times$ 150 mm (5 μm); flow rate 6 mL/min; detection UV 220 nm; room temperature; injection volume 200 μL per time; running time 15 mins per injection; automatic fraction collector mode peak-based.

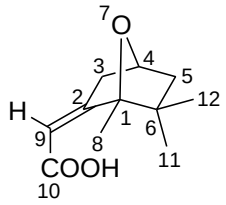
Table 2.13 Programme for separation of fraction Fr. B2-1 of *G. bicolor* by column chromatography over MCI gel

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	200
MeOH/H_2O	40:60	200
MeOH/H_2O	60:40	200
MeOH/H_2O	80:20	200
MeOH (clean up)	100	100

Table 2.14 Column chromatography fractionation of the fraction MeOH/H_2O 60:40 of *G. bicolor*

Mobile phase	Gradient elution	Solvent volume (mL)
CH_2Cl_2/MeOH	100:1	400
CH_2Cl_2/MeOH	100:2	400
CH_2Cl_2/MeOH	100:10	200

Analysis of its spectral data, together with comparison with data available in the literature,[160] allowed to elucidate the structure of the compound **150** and to identify it as ficusic acid.



150

Ficusic acid 150: white powder; ESI-MS m/z: 197 $[M+H]^+$; ^{1}H NMR δ ppm ($CDCl_3$, 300 MHz): 1.27 (3H, s, H-11), 1.31 (3H, s, H-12), 1.34 (1H, t, *J* = 12.1 Hz, H-5b), 1.51 (1H, t, *J* = 11.7 Hz, H-3b), 1.59 (3H, s, H-8), 2.04 (1H, ddd, *J* = 12.1, 4.0, 2.2 Hz, H-5a), 2.54 (1H, ddd, *J* = 11.7, 3.9, 2.2 Hz, H-3a), 4.06-4.19 (1H, m, H-4), 5.71 (1H, s, H-9). ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 181.5 (C-10), 172.0 (C-2), 113.1 (C-9), 86.9 (C-1), 64.9 (C-4), 49.7 (C-3), 47.9 (C-5), 35.2 (C-6), 30.0 (C-8), 25.6 (C-12), 25.1 (C-11).

2.2.3.2.5 Isolation of dehydrovomifoliol **151** and loliolide **152**

Fr. B2-2 (175 mg) was further subjected to column chromatography over MCI gel eluted by a gradient of water and methanol (20-100%) (Table 2.15). The 40-60% (methanol in water) eluent (93 mg) was combined for subsquent purification. Upon further silica gel column chromatography (petroleum ether/EtOAc, 10:1 to 1:1) (Table 2.16) and prep-HPLC (H_2O/CH_3CN, 75:25), two pure compounds **151** (Rt: 12.0-12.8 min., 0.5 mg) and **152** (Rt: 15.3-17.1 min., 4.0 mg) were obtained. The prep-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); flow rate 6 mL/min; detection UV 220 nm; room temperature; injection volume 150 μL per time; running time 20 mins per injection; automatic fraction collector mode peak-based.

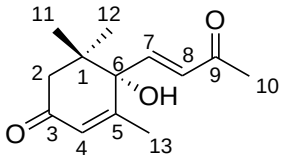
Table 2.15 Programme for separation of fraction Fr. B2-2 of *G. bicolor* by column chromatography over MCI gel

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	150
MeOH/H_2O	40:60	150
MeOH/H_2O	60:40	150
MeOH/H_2O	80:20	150
MeOH (clean up)	100	100

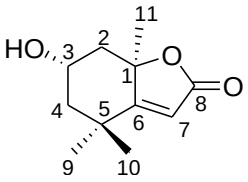
Table 2.16 Column chromatography fractionation of the fractions MeOH/H_2O 40:60 and 60:40 of *G. bicolor*

Mobile phase	Gradient elution	Solvent volume (mL)
Petroleum ether/EtOAc	10:1	330
Petroleum ether/EtOAc	8:1	360
Petroleum ether/EtOAc	4:1	500
Petroleum ether/EtOAc	2:1	300
Petroleum ether/EtOAc	1:1	200

Analysis of their spectral data, together with comparison with data available in the literatures,[161,162] allowed to elucidate the structure of compound **151** and **152** as dehydrovomifoliol and loliolide, respectively.



151



152

Dehydrovomifoliol 151: colorless gum; ESI-MS m/z: 223 $[M+H]^+$; ^1H NMR δ ppm ($CDCl_3$, 300 MHz): 1.03 (3H, s, H-12), 1.11 (3H, s, H-11), 1.89 (3H, s, H-13), 2.31 (3H, s, H-10), 2.34 (1H, d, *J* = 17.1 Hz, H-2a), 2.51 (1H, d, *J* = 17.1 Hz, H-2b), 5.96 (1H, s, H-4), 6.47 (1H, d, *J* = 16.0 Hz, H-8), 6.84 (1H, d, *J* = 16.0 Hz, H-7). ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 197.4 (C-3), 197.0 (C-9), 160.3 (C-5), 145.0 (C-7), 130.4 (C-8), 127.9 (C-4), 79.4 (C-6), 49.6 (C-2), 41.5 (C-1), 28.5 (C-10), 24.4 (C-11), 23.0 (C-12), 18.8 (C-13).

Loliolide 152: colorless needles; m.p. 148-149 °C; ESI-MS m/z: 197 $[M+H]^+$; ^1H NMR δ ppm ($CDCl_3$, 300 MHz): 1.27 (3H, s, H-9), 1.47 (3H, s, H-10), 1.53 (1H, dd, *J* = 14.3, 3.9 Hz, H-2a), 1.78 (1H, dd, *J* = 14.3, 3.9 Hz, H-4a), 1.79 (3H, s, H-11), 1.98 (1H, dt, *J* = 14.3, 2.8 Hz, H-2b), 2.47 (1H, dt, *J* = 14.3, 2.8 Hz, H-4b), 4.30-4.37 (1H, m, H-3), 5.70 (1H, s, H-7). ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 182.5 (C-6), 172.0 (C-8), 113.0 (C-7), 86.8 (C-1), 66.9 (C-3), 47.4 (C-2), 45.7 (C-4), 36.0 (C-5), 30.7 (C-9), 27.1 (C-11), 26.6 (C-10).

Noteworthy, the ^1H and ^{13}C NMR spectral data of compound **152**, which were in correspondence with loliolide,[162] were also nearly identical with those of a compound,

named pubinernoid A, which was isolated from *Schisandra pubescens* var. *pubinervis*.[163] The structural proposal for pubinernoid A, with the same molecular formula as loliolide, was (4*S**,6*R**)-4-hydroxy-4,8,8-trimethyl-9-oxabicyclo[4.2.1]non-1-en-3-one, with an atypical value of 183.2 ppm for the chemical shift of the keto group. However, upon comparing GC-MS spectral data with data available in the WILEY6N database, compound **152** was identified as loliolide.

The GC-MS conditions were: HP-5MS (crosslinked 5% PH ME siloxane) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and the gas carrier was helium, at 1 mL/min rate. The injector and MS-transfer line temperatures were maintained at 220 and 290 °C, respectively. Oven temperature was held at 40 °C for 3 min, raised to 180 °C at 2 °C/min and then to 250 °C at 10 °C/min (5 min hold). Electron impact mass spectra recorded at 40–500 mass range. An electron ionization system was used with ionization energy of 70 eV.

Loliolide 152: EIMS, m/z: 196(M^+, 10), 178(76), 163(43), 151(25), 140(44), 135(48), 111(100), 107(46), 95(31), 79(23), 67(22), 57(20), 43(54).

2.2.3.2.6 Isolation of vomifoliol **153** and boscialin **154**

Fr. B3 (655 mg) was fractionated with an automatic flash chromatography system on a reversed-phase column (C-18, 12 g) eluted by a gradient of water and methanol (50-100%) to get four fractions (Fr. B3-1~4). The automatic flash chromatography conditions were (Table 2.17 and 2.18):

Table 2.17 Automatic flash chromatography conditions of Fr. B3 of *G. bicolor*

Run conditions	
Cartridge:	Reveleris 12 g C18
Solvent A:	Water
Solvent B:	Methanol
Flow rate:	30 mL/min
UV1 wavelength:	220 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 2.18 Automatic flash chromatography gradient method of Fr. B3 of *G. bicolor*

Gradient Method		
Step	Time (min.)	%B
1	0	50
2	10	50
3	20	70
4	20	90
5	10	100
6	5	100

The collected fractions were concentrated by evaporation under reduced pressure with a rotavapor and after monitoring by TLC with UV detection, three combined fractions (Fr. B3-1~3) were obtained (Table 2.19).

Table 2.19 Column chromatography fractionation of Fr. B3 of *G. bicolor*

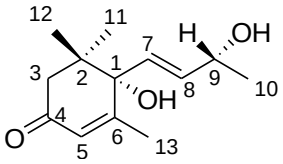
Fraction	Weight (mg)
Fr. B3-1	194
Fr. B3-2	305
Fr. B3-3	112

Fr. B3-2 (305 mg) was further subjected to column chromatography over MCI gel eluted by a gradient of water and methanol (20-100%) (Table 2.20). The 60% (methanol in water) eluent (92 mg) was collected for subsquent purification. A prep-HPLC method was developed over a reversed phase preparative column to yield two pure compounds **153** (Rt: 11.0-12.3 min, 1.2 mg) and **154** (Rt: 12.3-12.5 min, 0.8 mg). The prep-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); H_2O/CH_3CN (72:28) isocratic, flow rate 6 mL/min; detection UV 230 nm; room temperature; injection volume 200 μL per time; running time 22.5 mins per injection; automatic fraction collector mode peak-based.

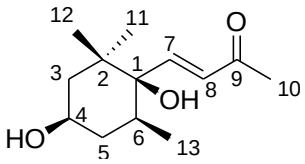
Table 2.20 Programme for separation of fraction Fr. B3-2 of *G. bicolor* by column chromatography over MCI gel

Mobile phase	Gradient elution	Solvent volume (mL)
$MeOH/H_2O$	20:80	200
$MeOH/H_2O$	40:60	200
$MeOH/H_2O$	60:40	300
$MeOH/H_2O$	80:20	200
MeOH (clean up)	100	100

Analysis of their spectral data, together with comparison with data available in the literatures,[164,165] allowed to elucidate the structure of compound **153** and **154** as vomifoliol and boscialin, respectively.



153



154

Vomifoliol 153: colorless gum; ESI-MS m/z: 283 $[M+OAc]^-$; ^1H NMR δ ppm ($CDCl_3$, 300 MHz): 1.01 (3H, s, H-11), 1.09 (3H, s, H-12), 1.31 (3H, d, *J* = 6.6 Hz, H-10), 1.90 (3H, d, *J* = 1.1 Hz, H-13), 2.25 (1H, d, *J* = 17.1 Hz, H-3a), 2.46 (1H, d, *J* = 17.1 Hz, H-3b), 4.37-4.47 (1H, m, H-9), 5.81 (1H, m, H-7), 5.85 (1H, m, H-8), 5.91 (1H, m, H-5). ^{13}C NMR δ ppm ($CDCl_3$,

75 MHz): 198.1 (C-4), 162.8 (C-6), 135.8 (C-8), 129.0 (C-7), 127.0 (C-5), 79.1 (C-1), 68.1 (C-9), 49.8 (C-3), 41.2 (C-2), 24.1 (C-11), 23.9 (C-10), 23.0 (C-12), 19.0 (C-13).

Boscialin 154: colorless gum; ESI-MS m/z: 285 $[M+OAc]^-$; ^{1}H NMR δ ppm ($CDCl_3$, 300 MHz): 0.81 (3H, d, *J* = 6.6 Hz, H-13), 0.88 (3H, s, H-11), 1.04 (3H, s, H-12), 1.33 (1H, m, H-5a), 1.50-1.71 (2H, m, H-3), 1.81-1.89 (1H, m, H-5b), 2.04 (1H, m, H-6), 2.29 (3H, s, H-10), 3.84-3.96 (1H, m, H-4), 6.37 (1H, d, *J* = 16.0 Hz, H-8), 6.76 (1H, d, *J* = 16.0 Hz, H-7). ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 197.9 (C-9), 150.7 (C-7), 130.4 (C-8), 77.1 (C-1), 66.5 (C-4), 45.1 (C-3), 40.0 (C-2), 39.0 (C-5), 34.0 (C-6), 28.3 (C-10), 25.2 (C-11), 24.7 (C-12), 16.0 (C-13).

2.2.3.3 Study of the ethyl acetate extract of the aerial parts of *G. bicolor*

The EtOAc extracted fraction (7.7 g) was adsorbed on 15 g of C18 silica and further fractionated by reversed phase column chromatography over C18 silica, eluted with water containing increasing concentrations of MeOH (Table 2.21). The fractions were collected in 100 mL tubes, after monitoring by TLC, a total of 5 different combined fractions (Fr. C1-5) were obtained (Table 2.22).

Table 2.21 Programme for fractionation of the EtOAc extract of *G. bicolor* by column chromatography

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	500
MeOH/H_2O	40:60	1200
MeOH/H_2O	60:40	1200
MeOH/H_2O	80:20	1000
MeOH (clean up)	100	500

Table 2.22 Column chromatography fractionation of the EtOAc extract of *G. bicolor*

Fraction	Weight (g)
Fr. C1	1.3
Fr. C2	0.7
Fr. C3	2.1
Fr. C4	2.9
Fr. C5	0.5

2.2.3.3.1 Isolation of 4-hydroxybenzoic acid **79**

Fr. C2 (724 mg) was submitted to fractionation with an automatic flash chromatography system on a reversed-phase column (C-18, 40 g) eluted by a gradient of water and methanol (20-100%). The automatic flash chromatography conditions were (Table 2.23 and 2.24):

Table 2.23 Automatic flash chromatography conditions of Fr. C2 of *G. bicolor*

Run conditions	
Cartridge:	Reveleris 40 g C18
Solvent A:	Water
Solvent B:	Methanol
Flow rate:	25 mL/min
UV1 wavelength:	254 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 2.24 Automatic flash chromatography gradient method of Fr. C2 of *G. bicolor*

Gradient Method		
Step	Time (min.)	%B
1	0	20
2	120	100

Following monitoring by TLC under UV, four different fractions (Fr. C2-1~4) were obtained (Table 2.25), after concentration by evaporation under reduced pressure with a rotavapor.

Table 2.25 Column chromatography fractionation of Fr. C2 of *G. bicolor*

Fraction	Weight (mg)
Fr. C2-1	72
Fr. C2-2	510
Fr. C2-3	54
Fr. C2-4	30

Fr. C2-2 (510 mg) was further subjected to column chromatography over silica gel eluted by a solvent gradient system consisting of $CHCl_3$-MeOH-H_2O (Table 2.26) to yield 5 fractions (Fr. C2-2-1~5, Table 2.27).

Table 2.26 Programme for fractionation of Fr. C2-2 of *G. bicolor* by column chromatography

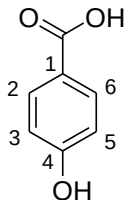
Mobile phase	Gradient elution	Solvent volume (mL)
$CHCl_3$/MeOH	100:1	300
$CHCl_3$/MeOH	20:1	400
$CHCl_3$/MeOH	10:1	700
$CHCl_3$/MeOH	6:1	700
$CHCl_3$/MeOH/H_2O	8:2:0.3	700
$CHCl_3$/MeOH/H_2O	7:3:0.5	700
$CHCl_3$/MeOH/H_2O	6:4:1	700

Table 2.27 Column chromatography fractionation of Fr. C2-2 of *G. bicolor*

Fraction	Weight (mg)
Fr. C2-2-1	--
Fr. C2-2-2	190
Fr. C2-2-3	--
Fr. C2-2-4	113
Fr. C2-2-5	--

Based on TLC, Fr. C2-2-4 (113 mg) was rechromatographed on a reversed-phase column with an automatic flash chromatography system eluted with an isocratic solvent system MeOH/H_2O (50:50). The collected fractions (56 mg), using a peak-based mode, were finally purified with silica gel column chromatography eluted by $CHCl_3$/MeOH (9:1) to yield a pure product (compound **79**, 13.6 mg).

Analysis of its spectral data, together with comparison with data available in the literature,[159] allowed to elucidate the structure of compound **79** as 4-hydroxybenzoic acid.



79

4-Hydroxybenzoic acid 79: white powder; m.p. 209-210 °C; ESI-MS m/z: 137 $[M-H]^-$; ^1H NMR δ ppm (DMSO-d_6, 300 MHz): 7.77 (2H, dd, *J* = 8.8, 3.9 Hz, H-2 and H-6), 6.80 (2H, dd, *J* = 8.8, 3.9 Hz, H-3 and H-5); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 167.7 (COOH), 162.1 (C-4), 132.1 (C-2 and C-6), 122.0 (C-1), 115.7 (C-3 and C-5).

2.2.3.3.2 Isolation of roseoside **155** and benzyl β-D-glucopyranoside **156**

Fr. C2-2-2 (190 mg) was further subjected to column chromatography over silica gel eluted by a gradient of $CHCl_3$ and methanol (100:1-6:1) (Table 2.28). Six fractions (Table 2.29) were obtained and two of them were selected for subsquent purification.

Table 2.28 Programme for separation of Fr. C2-2-2 of *G. bicolor*

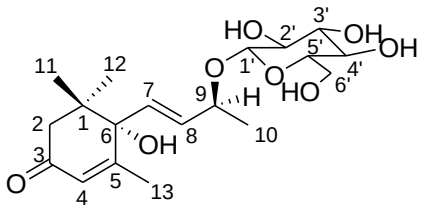
Mobile phase	Gradient elution	Solvent volume (mL)
$CHCl_3$/MeOH	100:1	500
$CHCl_3$/MeOH	100:2	500
$CHCl_3$/MeOH	100:5	500
$CHCl_3$/MeOH	100:10	500
$CHCl_3$/MeOH	6:1	300

Table 2.29 Column chromatography fractionation of Fr. C2-2-2 of *G. bicolor*

Fraction	Weight (mg)
Fr. C2-2-2-1	15
Fr. C2-2-2-2	13
Fr. C2-2-2-3	12
Fr. C2-2-2-4	20
Fr. C2-2-2-5	26
Fr. C2-2-2-6	35

Fr. C2-2-2-3 (12 mg) was finally purified by HPLC on a RP18 column to afford a product (compound **155**, 2.2 mg). The analytical HPLC conditions were the following: column, inertsil ODS-SP, 5 μm 4.6 × 250 mm, Shimadzu; elution, isocratic mode with MeOH/H_2O (36:64); flow rate, 0.8 mL/min; detection, UV 254 nm; eluted time, 10.1 min; running time, 15 min. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); elution, isocratic mode with MeOH/H_2O (36:64); flow rate, 6 mL/min; detection, UV 254 nm; room temperature; injection volume, 150 μL per time; running time, 18 mins per injection; automatic fraction collector mode peak-based. The sample was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. The eluates from 15.3-16.2 min were mixed and dried under high vacuum to afford compound **155**.

Analysis of the spectral data, together with comparison with data available in the literature,[166] allowed to elucidate the structure of compound **155** as (6*S*,9*S*)-roseoside.



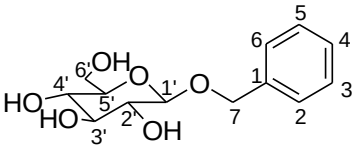
155

(6*S*,9*S*)-Roseoside 155: white powder; m.p. 98-99 °C; ESI-MS m/z: 445 $[M+OAc]^-$; ^1H NMR δ ppm (MeOH-d_4, 300 MHz): 0.93 (3H, s, H-11), 0.95 (3H, s, H-12), 1.20 (3H, d, *J* = 6.1 Hz, H-10), 1.86 (3H, d, *J* = 1.1 Hz, H-13), 2.08 (1H, d, *J* = 17.1 Hz, H-2a), 2.53 (1H, d, *J* = 17.1 Hz, H-2b), 3.02-3.26 (4H, m, H-2', 3', 4' and 5'), 3.54 (1H, dd, *J* = 12.1, 6.1 Hz, H-6'a), 3.77 (1H, dd, *J* = 12.1, 2.2 Hz, H-6'b), 4.18 (1H, d, *J* = 7.7 Hz, H-1'), 4.41-4.49 (1H, m, H-9), 5.64 (1H, dd, *J* = 16, 7.2 Hz, H-8), 5.79 (1H, s, H-4), 5.90 (1H, d, *J* = 16 Hz, H-7); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 201.3 (C-3), 167.1 (C-5), 133.8 (C-7), 133.7 (C-8), 127.1 (C-4), 101.2 (C-1'), 80.0 (C-6), 78.4 (C-3'), 78.2 (C-5'), 74.9 (C-2'), 74.6 (C-9), 71.7 (C-4'), 62.8 (C-6'), 50.8 (C-2), 42.4 (C-1), 24.7 (C-12), 23.5 (C-11), 22.2 (C-10), 19.6 (C-13).

Fr. C2-2-2-4 (20 mg) was finally purified by HPLC on a RP18 column to afford a pure product (compound **12**, 1.1 mg). The analytical HPLC conditions were the following: column, inertsil ODS-SP, 5μm 4.6 × 250mm, Shimadzu; elution, isocratic mode with

MeOH/H_2O (36:64); flow rate, 0.8 mL/min; detection, UV 254 nm; eluted time, 9.6 min; running time, 13 min. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); elution, isocratic mode with MeOH/H_2O (36:64); flow rate, 6 mL/min; detection, UV 254 nm; room temperature; injection volume, 150 μL per time; running time, 18 mins per injection; automatic fraction collector mode peak-based. The sample was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. The eluates from 14.5-15.0 min were mixed and dried under high vacuum to afford compound **156**.

Analysis of the spectral data, together with comparison with data available in literatures,[167,168] allowed to elucidate the structure of compound **156** as benzyl β-D-glucopyranoside.



156

Benzyl β-D-glucopyranoside 156: white powder; m.p. 114-115 °C; ESI-MS m/z: 329 $[M+OAc]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 3.25-3.35 (4H, m, H-2', 3', 4', 5'), 3.69 (1H, dd, *J* = 11.8, 5.5 Hz, H-6'a), 3.90 (1H, dd, *J* = 11.8, 1.9 Hz, H-6'b), 4.35 (1H, d, *J* = 7.7 Hz, H-1'), 4.66 (1H, d, *J* = 11.6 Hz, H-7a), 4.94 (1H, d, *J* = 11.6 Hz, H-7b), 7.24-7.44 (5H, m, H-2, 3, 4, 5, 6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 139.1 (C-1), 129.3 (C-3 and C-5), 129.2 (C-2 and C-6), 128.7 (C-4), 103.3 (C-1'), 78.1 (C-3' and C-5'), 75.1 (C-2'), 71.7 (C-4' and C-7), 62.8 (C-6').

2.2.3.3.3 Isolation of protocatechuic acid **157**

Fr. C3 (2.1 g) was split into four equal parts and each part was submitted to fractionation with an automatic flash chromatography system on a reversed-phase column (C-18, 40 g) eluted by a gradient of water and methanol (20-100%). The automatic flash chromatography conditions were (Table 2.30 and 2.31):

Table 2.30 Automatic flash chromatography conditions of Fr. C3 of *G. bicolor*

Run conditions	
Cartridge:	Reveleris 40 g C18
Solvent A:	Water
Solvent B:	Methanol
Flow rate:	25 mL/min
UV1 wavelength:	254 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 2.31 Automatic flash chromatography gradient method of Fr. C3 of *G. bicolor*

Gradient Method		
Step	Time (min.)	%B
1	0	20
2	120	100

Following monitoring by TLC under UV, four different fractions (Fr. C3-1~4) were obtained (Table 2.32), after concentration by evaporation under reduced pressure with a rotavapor.

Table 2.32 Column chromatography fractionation of Fr. C3 of *G. bicolor*

Fraction	Weight (mg)
Fr. C3-1	70
Fr. C3-2	1200
Fr. C3-3	318
Fr. C3-4	421

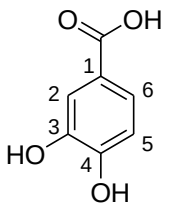
Fr. C3-1 (70 mg) was further subjected to column chromatography over Sephadex LH-20 gel eluted by an isocratic solvent system consisting of MeOH/H_2O (90:10) to yield three fractions (Fr. C3-1-1~3, Table 2.33).

Table 2.33 Column chromatography fractionation of Fr. C3-1 of *G. bicolor*

Fraction	Weight (mg)
Fr. C3-1-1	--
Fr. C3-1-2	46
Fr. C3-1-3	--

Fr. C3-1-2 (46 mg) was submitted to chromatography over silica gel eluted by an isocratic solvent system $CHCl_3$/MeOH (8:1) to afford a pure product (compound **157**, 12.5 mg).

Analysis of its spectral data, together with comparison with data available in the literature,[159] allowed to elucidate the structure of compound **157** as protocatechuic acid.



157

Protocatechuic acid 157: white powder; m.p. 194-195 °C; ESI-MS m/z: 153 $[M-H]^-$; ^1H NMR δ ppm (MeOH-d_4, 300 MHz): 7.34 (1H, s, H-2), 7.32 (1H, dd, *J* = 7.0, 1.7 Hz, H-6), 6.69 (1H, d, *J* = 7.0 Hz, H-5); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 168.9 (COOH), 150.2 (C-4), 144.8 (C-3), 122.6 (C-6), 121.8 (C-1), 116.4 (C-2), 114.4 (C-5).

2.2.3.3.4 Isolation of kaempferol **40**

Fr. C3-2 (1200 mg) was further subjected to column chromatography over silica gel eluted by a solvent gradient system consisting of $CHCl_3$-MeOH-H_2O (Table 2.34) to yield five fractions (Fr. C3-2-1~5, Table 2.35).

Table 2.34 Programme for fractionation of Fr. C3-2 of *G. bicolor* by column chromatography

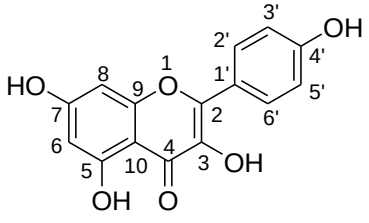
Mobile phase	Gradient elution	Solvent volume (mL)
$CHCl_3$/MeOH	100:1	600
$CHCl_3$/MeOH	20:1	1000
$CHCl_3$/MeOH	10:1	2000
$CHCl_3$/MeOH	4:1	1500
$CHCl_3$/MeOH/H_2O	8:2:0.3	1000
$CHCl_3$/MeOH/H_2O	7:3:0.5	800
$CHCl_3$/MeOH/H_2O	6:4:1	500

Table 2.35 Column chromatography fractionation of Fr. C3-2 of *G. bicolor*

Fraction	Weight (mg)
Fr. C3-2-1	65
Fr. C3-2-2	70
Fr. C3-2-3	200
Fr. C3-2-4	40
Fr. C3-2-5	130

Fr. C3-2-2 (70 mg) was submitted to chromatography over silica gel eluted by as isocratic solvent system $CHCl_3$/MeOH (10:1). The similar fractions were combined and additionally purified by column chromatography over Sephadex LH-20 gel eluted by MeOH/H_2O (80:20) to afford a pure yellowish product (compound **40**, 6.3 mg).

Analysis of its spectral data, together with comparison with data available in the literature,[169] allowed to elucidate the structure of compound **40** as kaempferol.



40

Kaempferol 40: yellow powder; m.p. 267-268 °C; ESI-MS m/z: 287 $[M+H]^+$; ^{1}H NMR δ ppm (DMSO-d_6, 300 MHz): 12.48 (1H, s, 5-OH), 10.80 (1H, brs, 4'-OH), 10.12 (1H, brs, 7-OH), 9.42 (1H, brs, 3-OH), 8.04 (2H, d, *J* = 9.1 Hz, H-2' and H-6'), 6.92 (2H, d, *J* = 9.1 Hz, H-3' and H-5'), 6.43 (1H, d, *J* = 2.2 Hz, H-8), 6.18 (1H, d, *J* = 2.2 Hz, H-6); ^{13}C NMR δ ppm (DMSO-d_6, 75 MHz): 176.4 (C-4), 164.5 (C-7), 161.3 (C-9), 159.8 (C-4'), 156.7 (C-5), 147.3 (C-2), 136.2 (C-3), 130.1 (C-2' and C-6'), 122.2 (C-1'), 116.0 (C-3' and C-5'), 103.5 (C-10),

98.8 (C-6), 94.0 (C-8).

2.2.3.3.5 Isolation of 2-phenylethyl β-D-glucopyranoside **158**

Fr. C3-2-3 (200 mg) was further subjected to column chromatography over silica gel eluted by a gradient of $CHCl_3$ and methanol (20:1-4:1) (Table 2.36) to afford four fractions (Table 2.37).

Table 2.36 Programme for separation of Fr. C3-2-3 of *G. bicolor*

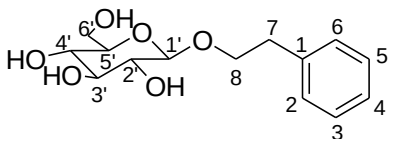
Mobile phase	Gradient elution	Solvent volume (mL)
$CHCl_3$/MeOH	20:1	500
$CHCl_3$/MeOH	10:1	800
$CHCl_3$/MeOH	8:1	1500
$CHCl_3$/MeOH	6:1	1000
$CHCl_3$/MeOH	4:1	500

Table 2.37 Column chromatography fractionation of Fr. C3-2-3 of *G. bicolor*

Fraction	Weight (mg)
Fr. C3-2-3-1	18
Fr. C3-2-3-2	27
Fr. C3-2-3-3	25
Fr. C3-2-3-4	24

Fr. C3-2-3-3 (25 mg) was finally purified by HPLC on a RP18 column to afford a pure product (compound **158**, 1.1 mg). The analytical HPLC conditions were the following: column, inertsil ODS-SP, 5 μm 4.6 × 250 mm, Shimadzu; elution, isocratic mode with MeOH/H_2O (35:65); flow rate, 0.8 mL/min; detection, UV 254 nm; eluted time, 12.3 min; running time, 15 min. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); elution, isocratic mode with MeOH/H_2O (35:65); flow rate, 6 mL/min; detection, UV 254 nm; room temperature; injection volume, 175 μL per time; running time, 20 mins per injection; automatic fraction collector mode peak-based. The sample was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. The eluates from 16.8-17.4 min were mixed and dried under high vacuum to afford compound **158**.

Analysis of their spectral data, together with comparison with data available in the literature,[168] allowed to elucidate the structure of compound **158** as 2-phenylethyl β-D-glucopyranoside.



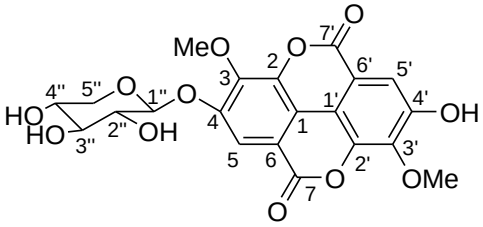
158

2-Phenylethyl β-D-glucopyranoside 158: white powder; m.p. 118-119 °C; ESI-MS m/z: 343 $[M+OAc]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 2.94 (2H, t-like, *J* = 7.2 Hz, H-7), 3.15-3.40 (4H, m, H-2', 3', 4', 5'), 3.60-3.79 (1H, m, H-8a), 3.66 (1H, dd, *J* = 11.5, 5.2 Hz, H-6'a), 3.86 (1H, dd, *J* = 11.5, 1.7 Hz, H-6'b), 4.05-4.13 (1H, m, H-8b), 4.30 (1H, d, *J* = 7.7 Hz, H-1'), 7.14-7.27 (5H, m, H-2, 3, 4, 5, 6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 140.1 (C-1), 130.0 (C-3 and C-5), 129.3 (C-2 and C-6), 127.2 (C-4), 104.4 (C-1'), 78.1 (C-5'), 78.0 (C-3'), 75.1 (C-2'), 71.7 (C-8), 71.6 (C-4'), 62.7 (C-6'), 37.2 (C-7).

2.2.3.3.6 Isolation of 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside **159**

Fr. C3-2-4 (40 mg) was submitted to chromatography over silica gel eluted by an isocratic solvent system $CHCl_3$/MeOH (6:1). The similar fractions were combined and additionally purified by repeated column chromatography over Sephadex LH-20 gel eluted by MeOH/H_2O (80:20) to afford a pure product (compound **159**, 4.1 mg).

Analysis of its spectral data, together with comparison with data available in the literature,[170] allowed to elucidate the structure of compound **159** as 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside.



159

3,3'-Di-O-methylellagic acid 4-O-β-D-xylopyranoside 159: white powder; m.p. 293-294 °C; ESI-MS m/z: 461 $[M-H]^-$; ^{1}H NMR δ ppm (DMSO-d_6, 300 MHz): 10.87 (1H, brs, 4'-OH), 7.72 (1H, s, H-5), 7.49 (1H, s, H-5'), 5.14 (1H, d, *J* = 6.6 Hz, H-1"), 4.06 (3H, s, 3-O$\underline{C}H_3$), 4.03 (3H, s, 3'-O$\underline{C}H_3$), 3.36 (5H, under solvent, H-2"~5"). ^{13}C NMR δ ppm (DMSO-d_6, 75 MHz): 159.0 (C-7 and C-7'), 153.4 (C-4'), 151.8 (C-4), 142.4 (C-3), 142.2 (C-2'), 141.5 (C-2), 140.7 (C-3'), 114.7 (C-1), 113.3 (C-6'), 112.4 (C-5), 112.4 (C-6), 112.2 (C-5'), 111.6 (C-1'), 102.3 (C-1"), 76.7 (C-3"), 73.6 (C-2"), 69.8 (C-4"), 66.3 (C-5"), 62.2 (3-O$\underline{C}H_3$), 61.6 (3'-O$\underline{C}H_3$).

2.3 Results and Discussion

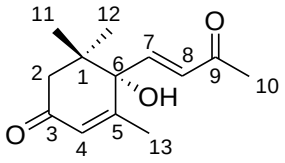
G. bicolor is native to the tropics of East Asia and has been cultivated as a popular vegetable in China, Japan and even in Europe. As reported in the literature[155], three anthocyanins were isolated and identified to be cyanidin 3-O-[β-D-glucopyranoside]-7-O-[6-O-(*E*)-(4-O-(6-O-(*E*)-caffeoyl-β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranoside]-3'-O-[6-O-(*E*)-caffeoyl-β-D-glucopyranoside] (bicolnin), cyanidin 3-O-[6-O-malonyl-β-D-glucopyranoside]-7-O-[6-O-(*E*)-(4-O-(6-O-(*E*)-caffeoyl-β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranoside]-3'-O-[6-O-(*E*)-caffeoyl-β-D-glucopyr-anoside] (rubrocinerarin) and cyanidin 3-O-[4,6-di-O-malonyl-β-D-glucopyranoside]-7-O-[6-O-(*E*)-(4-O-(6-O-(*E*)-caffeoyl-β-D-glu-

copyranosyl)-caffeoyl)-β-D-glucopyranoside]-3'-O-[6-O-(*E*)-caffeoyl-β-D-glucopyranoside] (bicolmalonin) by MS and NMR analyses.

In the present study, two terpenes (**150** and **152**), four megastigmane-type norisoprenoids (**151**, **153**, **154** and **155**), three glycosides (**156**, **158** and **159**), one flavonoid (**40**), three phenolic acids (**80**, **79** and **157**) and three other natural products (**148**, **78** and **149**) were isolated from the aerial parts of *G. bicolor*. All the compounds were characterized by spectrometric methods (NMR, MS). It should be noted that all these compounds, except 4-hydroxybenzoic acid (**79**) and kaempferol (**40**) were isolated for the first time from this plant, and no evidence could be found of previous reported presence of megastigmane-type norisoprenoids in the genus *Gynura*.

2.3.1 Megastigmane-type norisoprenoids from the aerial parts of *G. bicolor*

2.3.1.1 C13-norisoprenoid compound: dehydrovomifoliol **151**



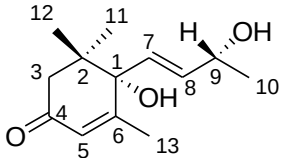
151

Compound **151** was obtained as a colorless gum. The ESI mass spectrum of compound **151** displayed a pseudomolecular ion peak at m/z 223 which may correspond to the fragment $[M+H]^+$. The molecular formula of $C_{13}H_{18}O_3$ was deduced. The ^1H NMR spectrum of **6** disclosed the presence of a vinyl proton at δ 5.96 (1H, s, H-4), two *E*-type olefinic protons at δ 6.47 (1H, d, *J* = 16.0 Hz, H-8) and 6.84 (1H, d, *J* = 16.0 Hz, H-7). In addition, protons of two gem-dimethyl groups at δ 1.11 (H-11) and 1.03 (H-12), protons of a methyl group at δ 1.89 (H-13) connected to a double bond, and a methyl group at δ 2.31 adjacent to a carbonyl group were observed. A geminal coupling system ascribed to the two protons of methylene at δ 2.51 (d, *J* = 17.1 Hz, H-2b) and 2.34 (d, *J* = 17.1 Hz, H-2a). Their chemical shift suggested that this methylene was connected to a carbonyl group and that the gem-dimethyl unit was positioned at C-1. On the other hand, the ^{13}C NMR spectrum of **151** exhibited two carbonyl signals at δ 197.4 (C-3) and 197.0 (C-9), four olefinic carbon signals at δ 127.9 (C-4), 130.4 (C-8), 145.0 (C-7), and 160.3 (C-5). Moreover, the observed signal at δ 79.4 (C-6) could be attributed to a quaternary carbon bearing the OH group. Based on all the above data, compound **151** was elucidated as the known (+)-dehydrovomifoliol. The assigned structure was in full agreement with data found in the literature (Table 2.38).[161]

Table 2.38 Comparison of characteristic ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) data of dehydrovomifoliol **151** with literature data (500/125 MHz, $CDCl_3$)[161]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1	--	41.5	--	41.42
2	2.34 (1H, d, *J* = 17.1 Hz), 2.51 (1H, d, *J* = 17.1 Hz)	49.6	2.33 (1H, d, *J* = 17.2 Hz), 2.49 (1H, d, *J* = 17.2 Hz)	49.59
3	--	197.4	--	197.17
4	5.96 (1H, s)	127.9	5.95 (1H, t-like)	127.87
5	--	160.3	--	160.08
6	--	79.4	--	79.32
7	6.84 (1H, d, *J* = 16.0 Hz)	145.0	6.82 (1H, d, *J* = 15.7 Hz)	144.85
8	6.47 (1H, d, *J* = 16.0 Hz)	130.4	6.45 (1H, d, *J* = 15.7 Hz)	130.40
9	--	197.0	--	196.76
10	2.31 (3H, s)	28.5	2.30 (3H, s)	28.36
11	1.11 (3H, s)	24.4	1.10 (3H, s)	24.33
12	1.03(3H, s)	23.0	1.02 (3H, s)	22.92
13	1.89 (3H, s)	18.8	1.88 (3H, d, *J* = 1.4 Hz)	18.60

2.3.1.2 C13-norisoprenoid compound: vomifoliol **153**



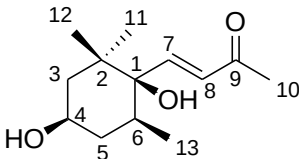
153

Compound **153** was obtained as a colorless gum. The ESI mass spectrum of compound **153** showed a molecular ion $[M+OAc]^-$ at m/z 283, suggesting a molecular formula of $C_{13}H_{20}O_3$. The ^{1}H NMR spectrum of **153** contained signals (2H, ca. δ 5.81-5.85, H-7 and H-8), which were assigned to the protons on a trans-disubstituted double bond. The signal of the methyl protons at δ 1.31 (3H, d, *J* = 6.6 Hz, H-10) coupled to the multiplet signal at ca. δ 4.42 (H-9) which also coupled to one of the ethylenic protons at δ 5.81-5.85. The presence of the moiety -CH=CH-CH(OH)CH_3 was established. Additionally, two doublets at 2.46 (H-3b) and 2.25 (H-3a) each with a coupling constant of 17.1 Hz suggested a nonequivalent methylene adjacent to the carbonyl and connected to a tetrasubstituted carbon. Besides, two gem-dimethyl groups at δ 1.01 (H-11) and 1.09 (H-12), a methyl group at δ 1.90 (H-13) connected to a double bond were observed. On the other hand, the ^{13}C NMR spectrum of **153** exhibited one carbonyl signal at δ 198.1 (C-4), four olefinic carbon signals at δ 127.0 (C-5), 129.0 (C-7), 135.8 (C-8), and 162.8 (C-6). The observed signal at δ 19.0 (C-13) suggested the methyl substituent located on the β-carbon of the α,β-unsaturated carbonyl system. The carbon signals at δ 79.1 (C-1) and δ 68.1 (C-9) could be attributed to two carbons bearing the OH group, respectively. These spectral data led to conclude the structure of compound **153** as the known vomifoliol. The spectral data of the compound isolated in the present study match well with those reported earlier (Table 2.39).[164]

Table 2.39 Comparison of characteristic ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) data of vomifoliol **153** with literature data (400/100 MHz, $CDCl_3$)[164]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		79.1		78.9
2		41.2		41.0
3	2.25 (1H, d, *J* = 17.1 Hz), 2.46 (1H, d, *J* = 17.1 Hz)	49.8	2.25 (1H, d, *J* = 16.9 Hz), 2.45 (1H, d, *J* = 16.9 Hz)	49.6
4		198.1		195.5
5	5.91 (1H, m)	127.0	5.90 (1H, m)	127.9
6		162.8		162.2
7	5.81 (1H, m)	129.0	5.81 (1H, m)	129.0
8	5.85 (1H, m)	135.8	5.84 (1H, m)	135.7
9	4.37-4.47 (1H, m)	68.1	4.41 (1H, m)	68.1
10	1.31 (3H, d, *J* = 6.6 Hz)	23.9	1.29 (3H, d, *J* = 6.4 Hz)	23.7
11	1.01 (3H, s)	24.1	1.01 (3H, s)	24.0
12	1.09 (3H, s)	23.0	1.08 (3H, s)	23.0
13	1.90 (3H, d, *J* = 1.1 Hz)	19.0	1.89 (3H, s)	18.8

2.3.1.3 C13-norisoprenoid compound: boscialin **154**



154

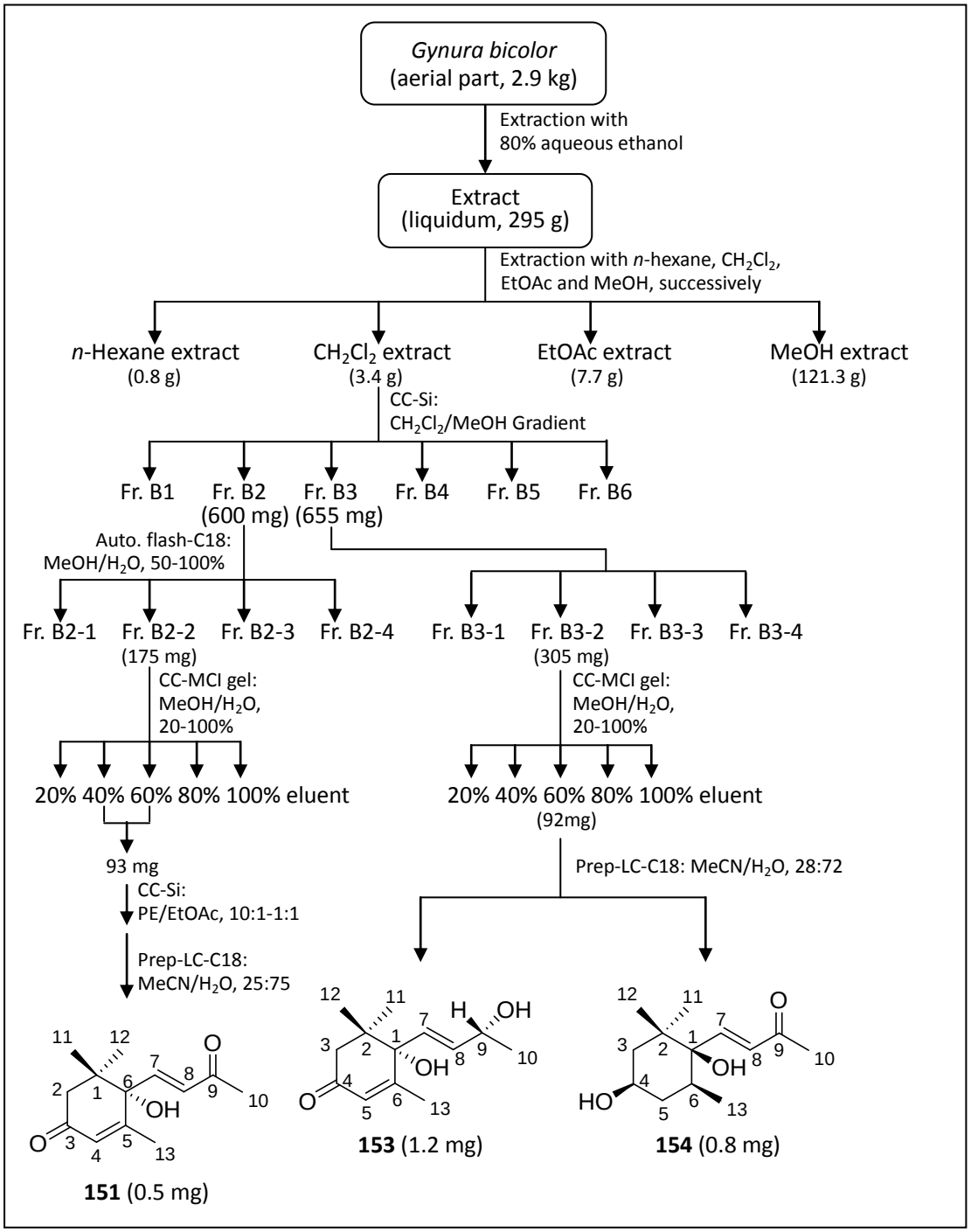
Compound **154** was obtained as a colorless gum. ESI mass spectrometric analysis of compound **154** showed a molecular ion $[M+OAc]^-$ at m/z 285 and a molecular ion $[M-H]^-$ at m/z 225, thus indicating a molecular formula of $C_{13}H_{22}O_3$. The ^{1}H NMR spectrum of **154** showed two singlets (3H) at δ 0.88 (H-11) and 1.04 (H-12) probably attached to a quaternary C-atom as well as the presence of a doublet (3H) at 0.81 (H-13, *J* = 6.6 Hz). An *E*-configurated double bond was deduced from the signals at δ 6.76 (1H, H-7) and δ 6.37 (1H, H-8) with a coupling constant of 16.0 Hz. The singlet (3H) at δ 2.29 (s, H-10) suggested a methyl adjacent to a carbonyl. The overlapping signals with signals from the solvent made it difficult to assign the rest of the proton signals. However, the ^{13}C NMR spectrum of **154** exhibited one carbonyl signal at δ 197.9 (C-9), two olefinic carbon signals at δ 150.7 (C-7) and 130.4 (C-8). The observed signal at δ 45.1 (C-3), 40.0 (C-2), 39.0 (C-5) and 34.0 (C-6) suggested a series of methylene, methine or quaternary C-atom signals. Analysis of the above spectral data, together with comparison with data available in the literature,[165] allowed to elucidate the structure of compound **154** as the known boscialin. The spectral data of the compound isolated in the present study match well with those reported earlier (Table 2.40).[165]

Table 2.40 Comparison of characteristic ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) data of boscialin **154** with literature data (400/100 MHz, $CDCl_3$)[165]

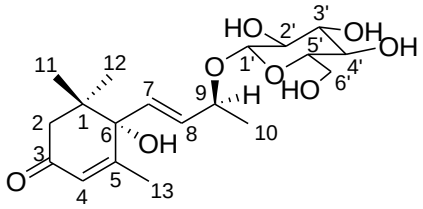
Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		77.1		77.0
2		40.0		39.9
3	1.50-1.71 (2H, m)	45.1	1.62 (t, *J* = 12 Hz, H_{ax}-3), 1.55 (H_{eq}-3)	45.1
4	3.84-3.96 (1H, m)	66.5	3.89 (1H, m)	66.4
5	1.33 (1H, m, H-5a), 1.81-1.89 (1H, m, H-5b)	39.0	1.34 (1H, q, *J* = 11.5 Hz, Hax-5), 1.83 (1H, br. dt, J = 12.5, 4 Hz, H_{eq}-5)	39.0
6	2.04 (1H, m)	34.0	2.08 (1H, m)	34.0
7	6.76 (1H, d, *J* = 16.0 Hz)	150.7	6.75 (1H, d, *J* = 16.0 Hz)	150.5
8	6.37 (1H, d, *J* = 16.0 Hz)	130.4	6.37 (1H, d, *J* = 16.0 Hz)	130.3
9		197.9		197.5
10	2.29 (3H, s)	28.3	2.28 (3H, s)	28.2
11	0.88 (3H, s)	25.2	0.88 (3H, s)	25.2
12	1.04 (3H, s)	24.7	1.04 (3H, s)	24.6
13	0.81 (3H, d, *J* = 6.6 Hz)	16.0	0.81 (3H, d, *J* = 6.5 Hz)	15.9

Scheme 2.1 describes the complete isolation of compounds **151**, **153** and **154** from the aerial parts of *G. bicolor*.

Scheme 2.1 The complete isolation of compounds **151**, **153** and **154** from *G. bicolor*



2.3.1.4 C13-norisoprenoid compound: (6*S*,9*S*)-roseoside **155**



155

Compound **155** was obtained as an amorphous powder. The ESI mass spectrum of **155** showed an ion peak at m/z 445, which corresponded to $[M+OAc]^-$ and suggested a molecular formula of $C_{19}H_{30}O_8$. In the ^1H NMR spectral data of compound **155**, two olefinic protons in the side chain were confirmed by signals at δ 5.90 (H-7) and 5.64 (H-8) with *J* = 16 Hz indicating the trans configuration. The signal at δ 5.79 was assignable to a vinyl proton. The two methyl singlets at δ 0.95 and 0.93 were assigned to gem-dimethyl groups on a quaternary carbon. The signal (3H) at δ 1.86 corresponded to a methyl next to a double bond. A doublet signal for three protons at δ 1.20 could be attributed to a methyl proton (H-10) linked to an oxygenated methine at δ 4.45 (1H, m, H-9). Proton peaks at δ 2.53 (d, *J* = 17.1 Hz, H-2b) and 2.08 (d, *J* = 17.1 Hz, H-2a) were geminally coupled signals from a methylene attached to a carbonyl group. In the sugar part, an anomeric proton appeared at δ 4.18 (d, *J* = 7.7 Hz, H-1') and its configuration could be deduced as β type. On the other hand, the ^{13}C NMR spectrum exhibited a carbonyl signal at δ 201.3 (C-3), four olefinic carbon signals at 133.8 (C-7), 133.7 (C-8), 167.1 (C-5) and 127.1 (C-4), two oxygenated carbon signals at δ 74.6 (C-9) and 80.0 (C-6). Moreover, the sugar moiety was identified as D-glucopyranoside when compared with the literature.[171] Thus, the structure of **155** was designated to roseoside.

Although most natural roseosides have the (6*S*)-configuration, (6*R*)-configurated roseoside has been isolated as well.[171] As reported in the literature, ^1H and ^{13}C NMR spectra of those epimers are so similar to each other that confirmation of stereochemistries of the natural glucosides from spectral data is not easy. The stereochemistry of C-9 was determined by comparing the chemical shift with reported data, which showed different signals at δ 77.0 and 74.7 for the 9*R* and 9*S* isomers, respectively.[172] In the latter paper, the configuration of C-9 (δ 74.6) was assigned as *S*. In addition, after analysis of the chemical shift differences of the present isolated roseoside and roseosides resulting from stereocontrolled synthesis, compound **155** was tentatively determined to be (6*S*,9*S*)-roseoside.[173]

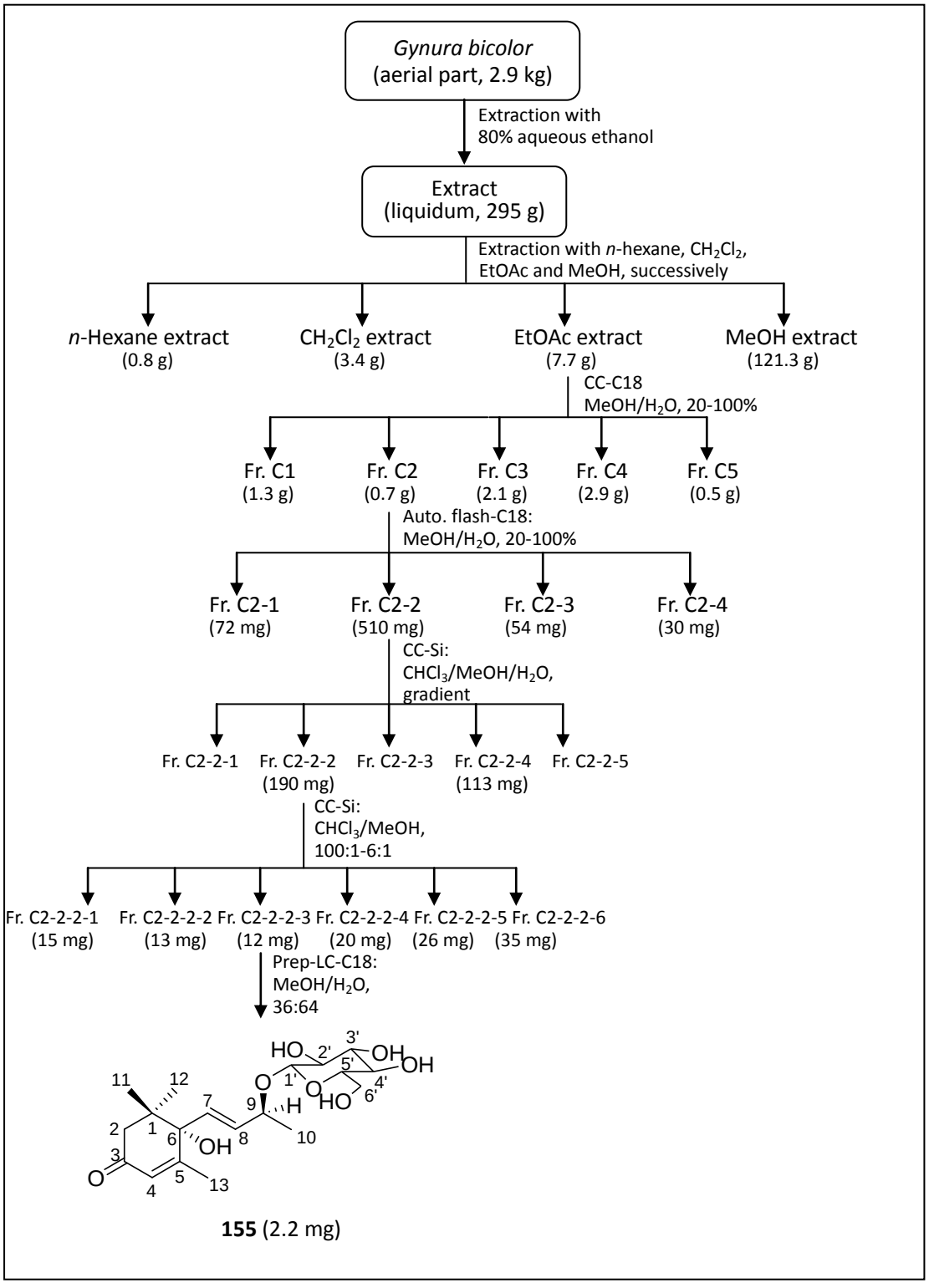
The spectral data of the compound isolated in the present study match well with those reported earlier. The comparison of characteristic NMR data of compound **155** with literature data is listed in the Table 2.40.[166]

Table 2.40 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of (6*S*,9*S*)-roseoside **155** with literature data (400/100 MHz, CD_3OD)[166]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		42.4		42.4
2	2.08 (1H, d, *J* = 17.1 Hz), 2.53 (1H, d, *J* = 17.1 Hz)	50.8	2.10 (1H, d, *J* = 17.1 Hz) 2.54 (1H, d, *J* = 17.1 Hz)	50.8
3		201.3		201.3
4	5.79 (1H, s)	127.1	5.80 (1H, s)	127.1
5		167.1		167.1
6		80.0		80.0
7	5.90 (1H, d, *J* = 16 Hz)	133.8	5.91 (1H, d, *J* = 15.6 Hz)	133.8
8	5.64 (1H, dd, *J* = 16, 7.2 Hz)	133.7	5.63 (1H, dd, *J* = 15.6, 7.3 Hz)	133.7
9	4.41-4.49 (1H, m),	74.6	4.46 (1H, m)	74.6
10	1.20 (3H, d, *J* = 6.1 Hz)	22.2	1.22 (3H, d, *J* = 6.3 Hz)	22.2
11	0.93 (3H, s)	23.5	0.95 (3H, s)	23.5
12	0.95 (3H, s)	24.7	0.97 (3H, s)	24.7
13	1.86 (3H, d, *J* = 1.1 Hz)	19.6	1.87 (3H, d, *J* = 1.5 Hz)	19.6
1’	4.18 (1H, d, *J* = 7.7 Hz)	101.2	4.20 (1H, d, *J* = 7.8 Hz)	101.2
2’		74.9		74.9
3’	3.02-3.26 (4H, m, H-2’~5’)	78.4	3.06-3.38 (4H, m, H-2’~5’)	78.4
4’		71.7		71.7
5’		78.2		78.2
6’	3.54 (1H, dd, *J* = 12.1, 6.1 Hz), 3.77 (1H, dd, *J* = 12.1, 2.2 Hz)	62.8	3.56 (1H,dd, *J* = 11.7, 6.3 Hz), 3.78 (1H, dd, *J* = 11.7, 2.0 Hz)	62.8

Scheme 2.2 describes the complete isolation of (6*S*,9*S*)-roseoside **155** from the aerial parts of *G. bicolor*.

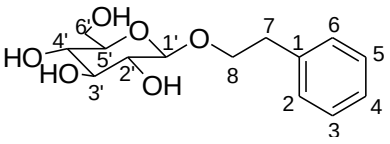
Scheme 2.2 The complete isolation of (6*S*,9*S*)-roseoside **155** from *G. bicolor*



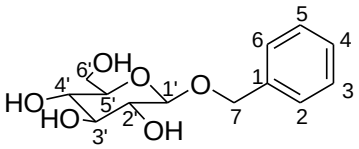
155 (2.2 mg)

2.3.2 Glycosides from the aerial parts of *G. bicolor*

2.3.2.1 Glycoside compounds: 2-phenylethyl β-D-glucopyranoside **158** and benzyl β-D-glucopyranoside **156**



158



156

Compound **158** was isolated as a white powder. The ESI mass spectrum of **158** showed an ion peak at m/z 343, two ion peaks at m/z 319/321, which corresponded to $[M+OAc]^-$ and $[M+Cl]^-$, suggesting a molecular formula of $C_{14}H_{20}O_6$. The ^1H NMR spectrum exhibited a triplet signal at δ 2.94 (2H, *J* = 7.2 Hz, H-7) which could result from benzylic methylene protons. The proton signals from a sugar moiety were seen in the spectrum, moreover, the doublet signal due to an anomeric proton appeared at δ 4.30 (1H, d, *J* = 7.7 Hz, H-1') and its configuration was confirmed as β type. The aromatic protons at δ 7.14-7.27 (5H, m, H-2~6) suggested a mono-substituted benzene ring in the molecule. The ^{13}C NMR spectrum exhibited two methylene carbon signals at δ 37.2 (C-7) and 71.7 (C-8). Comparison with data available in the literature allowed to elucidate the sugar moiety of the compound **158** as D-glucopyranoside.[167] Moreover, these data allowed to conclude the structure of this compound to be 2-phenylethyl β-D-glucopyranoside.

Compound **156** was isolated as a white powder. The ESI mass spectrum of **156** showed an ion peak at m/z 329, two ion peaks at m/z 305/307, which corresponded to $[M+OAc]^-$ and $[M+Cl]^-$, suggesting a molecular formula of $C_{13}H_{18}O_6$. The ^1H and ^{13}C NMR data were almost the same as those of compound **158**, except for the presence of a Ph-CH_2 group at δ 2.94 in compound **158**. Based on further comparison with published data,[168] the structure of compound **156** was identified as benzyl β-D-glucopyranoside.

The spectral data of the compounds isolated in the present study match well with those reported earlier.[167,168] The comparison of characteristic NMR data of compounds **158** and **156** with literature data is listed in the Table 2.41 and 2.42.[168]

Table 2.41 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of 2-phenylethyl β-D-glucopyranoside **158** with literature data (300/75 MHz, CD_3OD)[168]

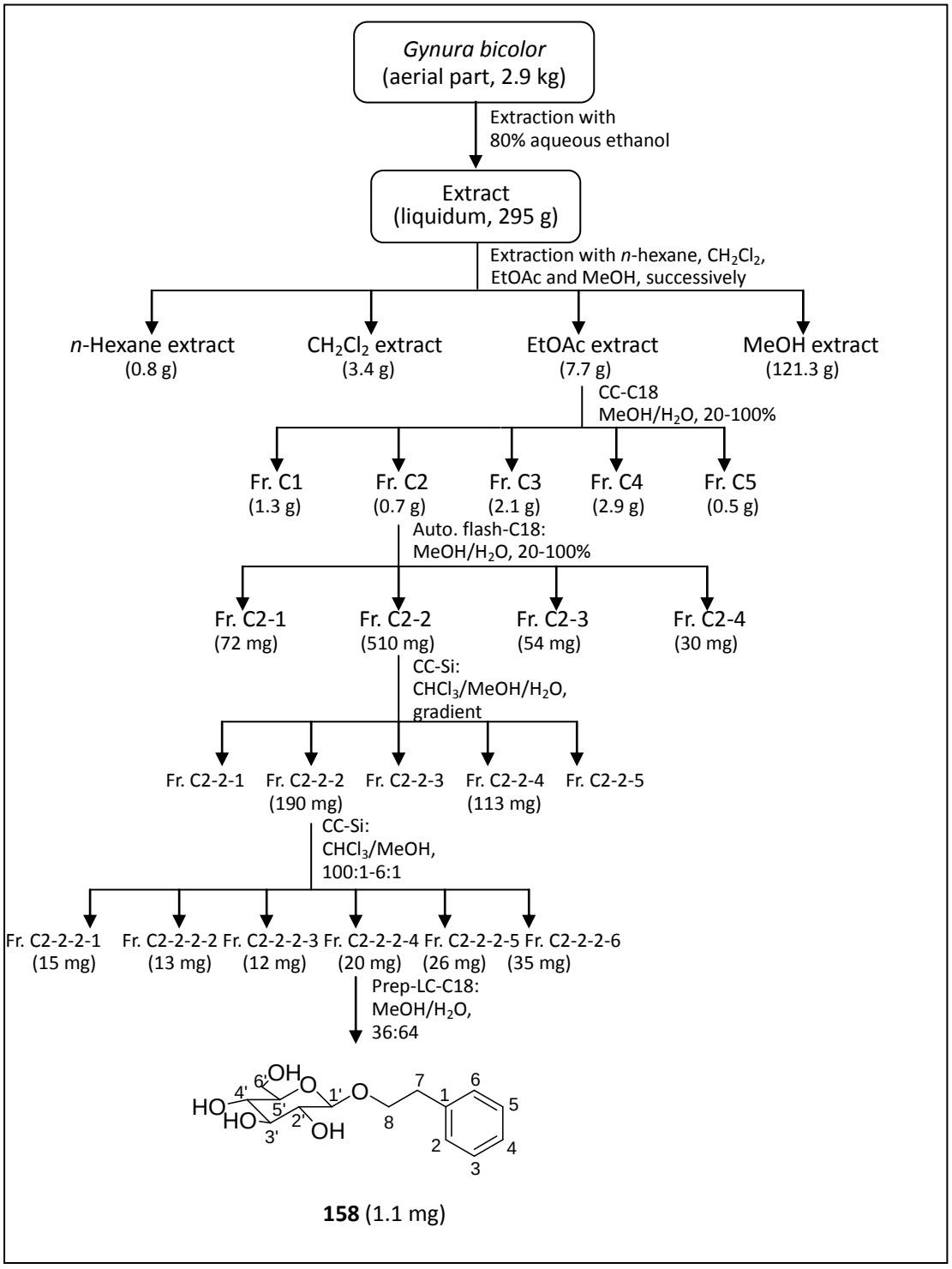
Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		140.1		140.0
2		129.3		129.3
3		130.0		130.0
4	7.14-7.27 (5H, m, H-2~6)	127.2	7.15-7.26 (5H, m, Ph)	127.1
5		130.0		130.0
6		129.3		129.3
7	2.94 (2H, t-like, *J* = 7.2 Hz)	37.2	2.93 (2H, "t")	37.2
8	3.60-3.79 (1H, m, H-8a) 4.05-4.13 (1H, m, H-8b)	71.7	3.59-3.79 (1H, m, H-8a) 4.05-4.13 (1H, m,H-8b)	71.7
1'	4.30 (1H, d, *J* = 7.7 Hz)	104.4	4.30 (1H, d, *J* = 7.7 Hz)	104.3
2'		75.1		75.0
3'	3.15-3.40 (4H, m, H-2'~5')	78.0	3.16-3.39 (4H, m, H-2'~5')	77.9
4'		71.6		71.6
5'		78.1		78.0
6'	3.66 (1H, dd, *J* = 11.5, 5.2 Hz, H-6'a), 3.86 (1H, dd, *J* = 11.5, 1.7 Hz, H-6'b)	62.7	3.65 (1H, dd, *J* = 12.0, 5.5 Hz, H-6'a), 3.86 (1H, dd, *J* = 12.0, 2.0 Hz, H-6'b)	62.7

Table 2.42 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of benzyl β-D-glucopyranoside **156** with literature data (300/75 MHz, CD_3OD)[168]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		139.1		139.0
2		129.2		129.2
3		129.3		129.25
4	7.24-7.44 (5H, m, H-2~6)	128.7	7.23-7.43 (5H, m, Ph)	128.7
5		129.3		129.25
6		129.2		129.2
7	4.66 (1H, d, *J* = 11.6 Hz, H-7a), 4.94 (1H, d, *J* = 11.6 Hz, H-7b)	71.7	4.65 (1H, d, *J* = 11.8 Hz,H-7a), 4.93 (1H, d, *J* = 11.8 Hz, H-7b)	71.7
1'	4.35 (1H, d, *J* = 7.7 Hz)	103.3	4.35 (1H, d, *J* = 7.6 Hz)	103.2
2'		75.1		75.1
3'	3.25-3.35 (4H, m, H-2'~5')	78.1	3.22-3.38 (4H, m, H-2'~5')	78.0
4'		71.7		71.6
5'		78.1		78.05
6'	3.69 (1H, dd, *J* = 11.8, 5.5 Hz, H-6'a), 3.90 (1H, dd, *J* = 11.8, 1.9 Hz, H-6'b)	62.8	3.69 (1H, dd, *J* = 12.0, 5.5 Hz, H-6'a), 3.89 (1h, dd, *J* = 12.0, 2.1 Hz, H-6'b)	62.8

Scheme 2.3 describes the complete isolation of 2-phenylethyl β-D-glucopyranoside **158** from the aerial parts of *G. bicolor*.

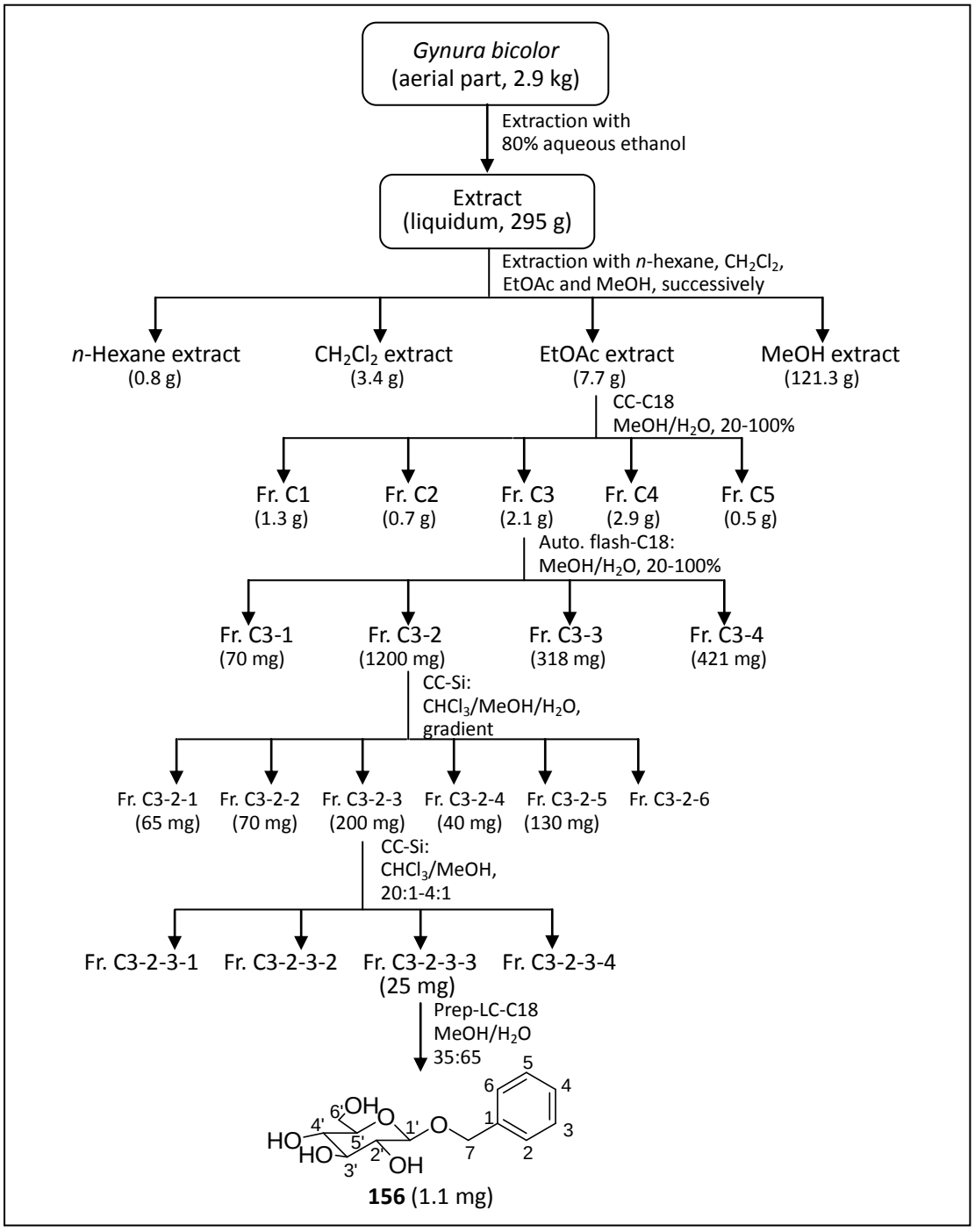
Scheme 2.3 The complete isolation of 2-phenylethyl β-D-glucopyranoside **158** from *G. bicolor*



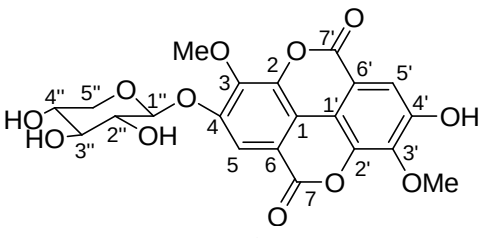
158 (1.1 mg)

Scheme 2.4 describes the complete isolation of benzyl β-D-glucopyranoside **156** from the aerial parts of *G. bicolor*.

Scheme 2.4 The complete isolation of benzyl β-D-glucopyranoside **156** from *G. bicolor*



2.3.2.2 Ellagic acid derivatives: 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside **159**



159

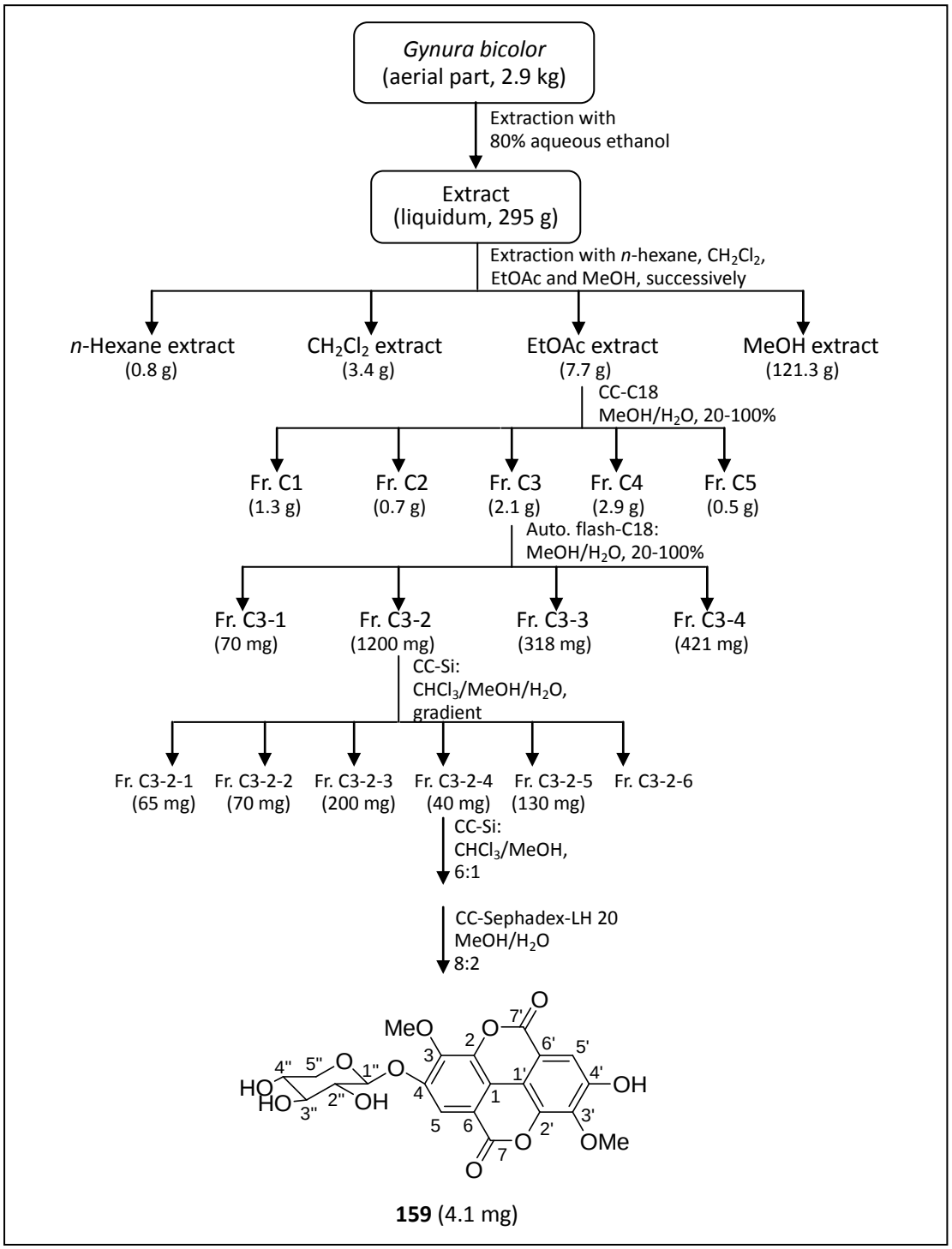
Compound **159** was obtained as a white powder and its molecular formula $C_{21}H_{18}O_{12}$ was deduced by the ESI mass spectrum (m/z 461 $[M-H]^-$). The ^1H NMR spectrum showed two singlets at δ 7.72 and 7.49 assignable to aromatic protons from the ellagic acid skeleton. An anomeric proton at δ 5.14 (1H, d, H-1") indicated a sugar moiety and its configuration was proposed to be β type on the basis of the coupling constant of 6.6 Hz. Two methoxy groups were observed as well at δ 4.06 (3H, s) and 4.03 (3H, s). In the ^{13}C NMR spectrum, two lactone carbonyls at δ 159.0 (C-7 and C-7') and 12 aromatic carbon signals could be due to ellagic acid. The sugar part of the compound was determined as a xylose moiety by comparison with published data.[170] Two remaining methoxy carbon signals appeared at 61.6 (3'-O<u>C</u>H_3) and 62.2 (3-O<u>C</u>H_3) suggesting two *ortho*-disubstituted methoxy groups in the structure. As consequence, the xylose was assigned to be linked with C-4 of ellagic acid. These data, together with comparison with data available in the literature,[170] allowed to elucidate the structure of compound **159** as the known 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside. The spectral data of the compound isolated in the present study match well with those reported earlier (Table 2.43).[170]

Table 2.43 Comparison of characteristic ^1H NMR (300 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6) data of 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside **159** with literature data (300/75 MHz, DMSO-d_6)[170]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1		114.7		114.1
2		141.5		140.8
3		142.4		141.8
4		151.8		151.1
5	7.72 (1H, s)	112.4	7.74 (1H, s)	111.8
6		112.4		111.8
7		159.0		158.2
1'		111.6		111.0
2'		142.2		141.5
3'		140.7		140.1
4'	10.87 (1H, brs)	153.4		152.7
5'	7.49 (1H, s)	112.2	7.52 (1H, s)	111.4
6'		113.3		112.7
7'		159.0		158.3
1"	5.14 (1H, d, *J* = 6.6 Hz)	102.3	5.14 (1H, d, *J* =7.2 Hz)	101.6
2"		73.6	3.33	72.9
3"	3.36 (4H, under solvent)	76.7	3.32	76.0
4"		69.8	3.38	69.1
5"		66.3	3.82(dd, *J* = 10.0, 4.0 Hz))	65.7
3-O<u>C</u>H_3	4.06 (3H, s)	62.2	4.06	61.5
3'-O<u>C</u>H_3	4.03 (3H, s)	61.6	4.03	60.9

Scheme 2.5 describes the complete isolation of 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside **159** from the aerial parts of *G. bicolor*.

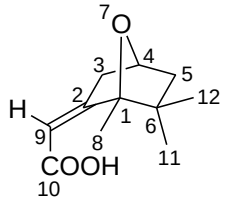
Scheme 2.5 The complete isolation of 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside **159** from *G. bicolor*



159 (4.1 mg)

2.3.3 Terpenes from the aerial parts of *G. bicolor*

2.3.3.1 Monoterpene compound: Ficusic acid **150**



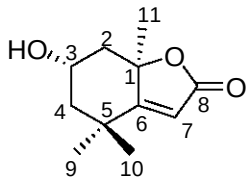
150

Compound **150** was isolated as a white powder. The ESI mass spectrum showed a molecular ion $[M+H]^+$ at m/z 197, suggesting a molecular formula of $C_{11}H_{16}O_3$. The ^1H NMR spectrum exhibited two geminal methyl singlets at δ 1.27 (3H, s, H-11), 1.31 (3H, s, H-12) and a methyl singlet at δ 1.59 (3H, s, H-8). The signal at 5.71 (1H, s, H-9) was attributable to an olefinic proton attached to one of the double bond carbons conjugated with the carboxylic acid. The signals of two methylene groups appeared at 2.54 (1H, ddd, *J* = 11.7, 3.9, 2.2 Hz, H-3a), 1.51 (1H, t, *J* = 11.7 Hz, H-3b), 2.04 (1H, ddd, *J* = 12.1, 4.0, 2.2 Hz, H-5a) and 1.34 (1H, t, *J* = 12.1 Hz, H-5b), respectively. In those methylene signals, H-3a and H-5a exhibited a W-type coupling (*J* = 2.2 Hz) as well. The methine proton signal at ca. 4.10 (1H, m, H-4) was assigned to be geminal to an ether linkage and situated between two methylene groups (H-3 and H-5). In the ^{13}C NMR spectrum, three signals at 181.5 (C-10), 172.0 (C-2) and 113.1 (C-9) were assigned to a carboxylic acid conjugated with a double bond. These data, together with comparison with data available in the literature,[160] allowed to elucidate the structure of compound **150** as the known ficusic acid. The spectral data of compound **150** isolated in the present study match well with those reported earlier (Table 2.44).[160]

Table 2.44 Comparison of characteristic ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) data of ficusic acid **150** with literature data (600/150 MHz, $CDCl_3$)[160]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1		86.9		86.4
2		172.0		171.5
3	1.51 (1H, t, *J* = 11.7 Hz, H-3b), 2.54 (1H, ddd, *J* = 11.7, 3.9, 2.2 Hz, H-3a)	49.7	2.54 (1H, ddd, *J* = 11.9, 3.7, 2.3 Hz), 2.04 (1H, ddd, *J* = 12.8, 4.1, 2.3 Hz)	49.8
4	4.06-4.19 (1H, m)	64.9	4.14 (1H, m)	65.1
5	1.34 (1H, t, *J* = 12.1 Hz, H-5b), 2.04 (1H, ddd, *J* = 12.1, 4.0, 2.2 Hz, H-5a)	47.9	1.51 (1H, t, *J* = 11.9 Hz), 1.33 (1H, t, *J* = 12.4 Hz)	47.9
6		35.2		35.0
8	1.59 (3H, s)	30.0	1.59 (3H, s)	30.0
9	5.71 (1H, s)	113.1	5.72 (1H, s)	113.3
10		181.5		180.7
11	1.27 (3H, s)	25.1	1.27 (3H, s)	25.1
12	1.31 (3H, s)	25.6	1.32 (3H, s)	25.6

2.3.3.2 Monoterpene compound: loliolide **152**



152

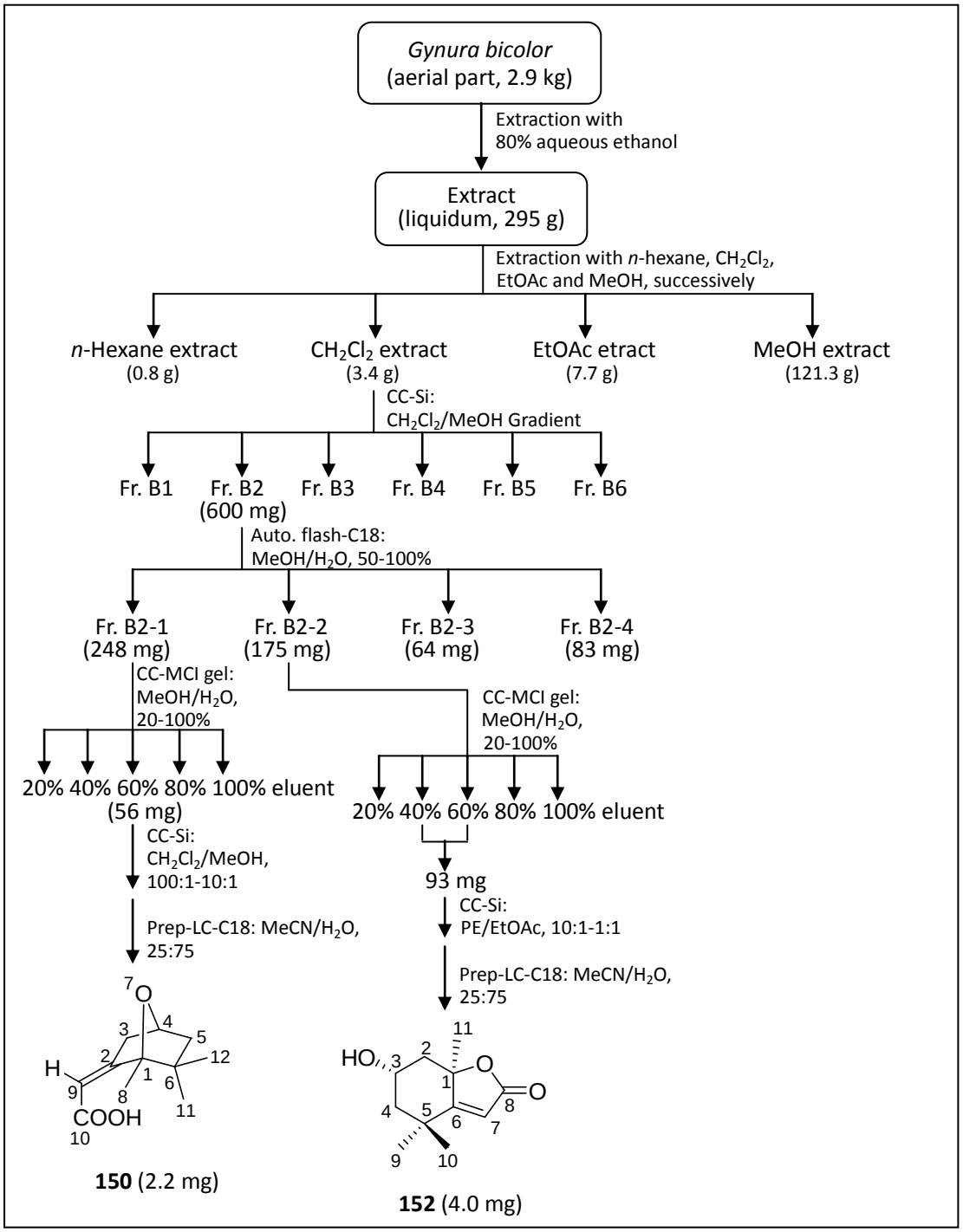
Compound **152** was obtained as colorless needles. A quasi-molecular ion peak at m/z 197 $[M+H]^+$ was observed in the ESI mass spectrum, suggesting that the molecular formula of **7** was $C_{11}H_{16}O_3$. In the ^1H NMR spectrum, three singlets at 1.27 (3H, s, H-9), 1.47 (3H, s, H-10) and 1.79 (3H, s, H-11) were observed, which indicated the presence of three methyl groups. In the olefinic region, only one sharp singlet at 5.70 appeared. The signals of two methylene groups were observed at 1.53 (1H, dd, *J* = 14.3, 3.9 Hz, H-2a), 1.98 (1H, dt, *J* = 14.9, 2.8 Hz, H-2b), 1.78 (1H, dd, *J* = 13.5, 4.1 Hz, H-4a) and 2.47 (1H, dt, *J* = 14.3, 2.8 Hz, H-4b) as well as one oxygenated methine at ca. 4.34, suggesting a CH_2-CH(OH)-CH_2 group of compound **152**. In addition, a carbonyl function (182.5, C-6), one trisubstituted double bond (172.0, C-8 and 113.0, C-7), one oxygenated quaternary carbon (86.8, C-1), one oxygenated methine (66.9, C-3) from the ^{13}C NMR spectrum, could be assigned also. Compared with the previous literature data reported (Table 2.45), [162] compound **152** was identified as the known loliolide.

Table 2.45 Comparison of characteristic ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) data of loliolide **152** with literature data (300/75 MHz, $CDCl_3$)[162]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1		86.8		86.7
2	1.53 (1H, dd, *J* = 14.3, 3.9 Hz, H-2a), 1.98 (1H, dt, *J* = 14.3, 2.8 Hz, H-2b)	47.4	1.50 (1H, dd, *J* = 15.0, 3.6 Hz) 1.94 (1H, dt, *J* = 14.7, 3.0 Hz)	47.3
3	4.30-4.37 (1H, m)	66.9	4.31 (1H, quintet, *J* = 3.6 Hz)	66.8
4	1.78 (1H, dd, *J* = 14.3, 3.9 Hz, H-4a), 2.47 (1H, dt, *J* = 14.3, 2.8 Hz, H-4b)	45.7	1.78 (1H, dd, *J* = 13.2, 3.9 Hz) 2.43 (1H, dt, *J* = 14.1, 3.0 Hz)	45.6
5		36.0		35.9
6		182.5		182.4
7	5.70 (1H, s)	113.0	5.67 (1H, s)	112.9
8		172.0		171.9
9	1.27 (3H, s)	30.7	1.25 (3H, s)	30.6
10	1.47 (3H, s)	26.6	1.45 (3H, s)	26.5
11	1.79 (3H, s)	27.1	1.76 (3H, s)	27.0

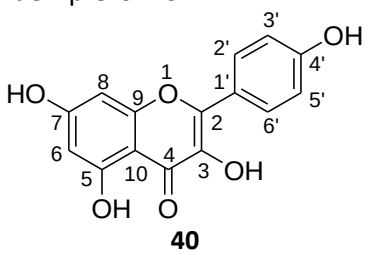
Scheme 2.6 describes the complete isolation of ficusic acid **150** and loliolide **152** from the aerial parts of *G. bicolor*.

Scheme 2.6 The complete isolation of ficusic acid **150** and loliolide **152** from *G. bicolor*



2.3.4 Flavonoids from the aerial parts of *G. bicolor*

2.3.4.1 Flavonoid compound: kaempferol **40**



40

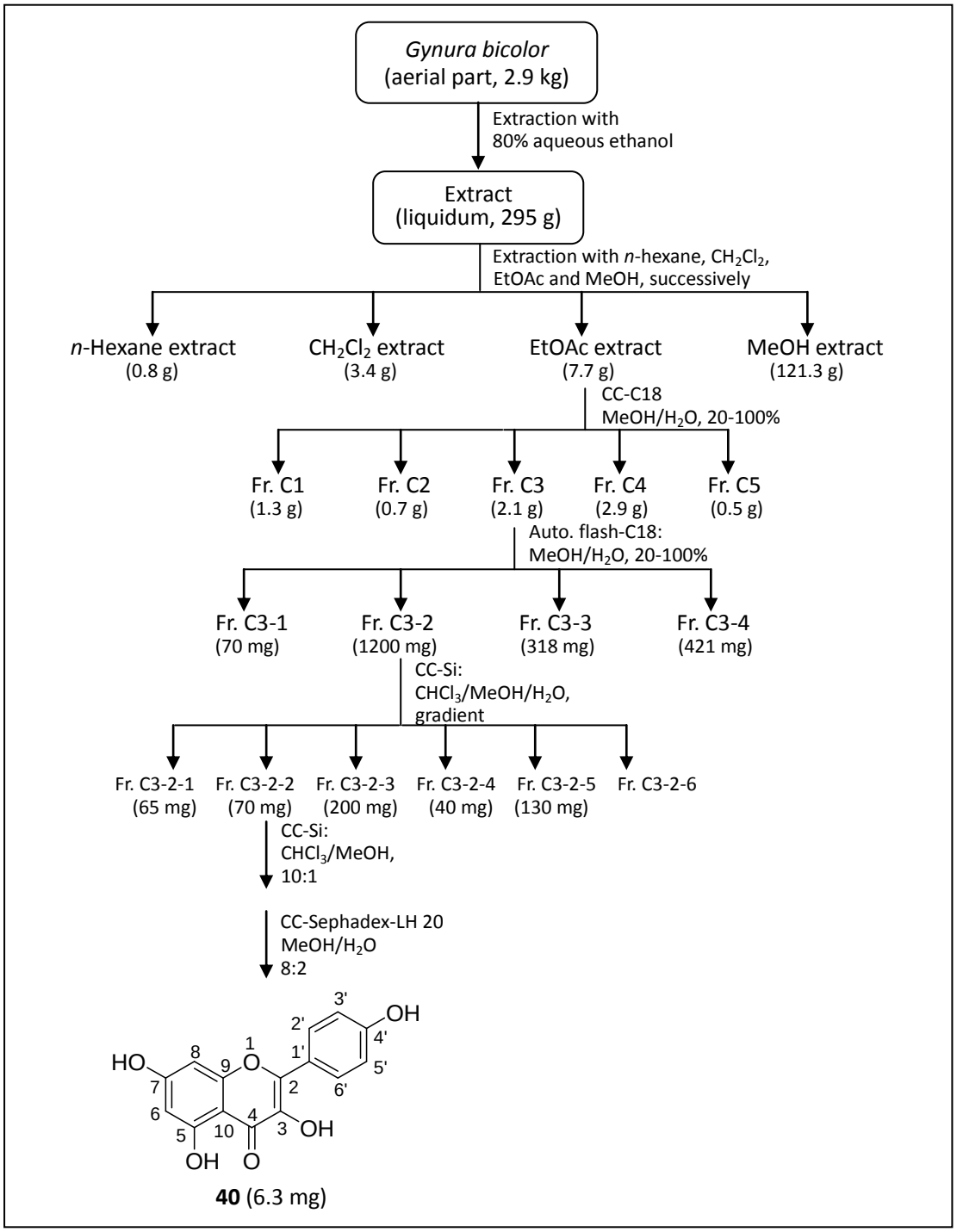
Compound **40** was obtained as a yellow powder. In the ESI mass spectrum, a quasi-molecular ion peak $[M-H]^-$ at *m/z* 285 was observed suggesting the molecular formula of **40** was $C_{15}H_{10}O_6$. The ^1H NMR spectrum showed two doublets at δ 6.18 (1H, d, *J* = 2.2 Hz, H-6) and 6.43 (1H, d, *J* = 2.2 Hz, H-8) corresponding to the meta-coupled protons on A-ring of a flavonoid. In addition, a typical A_2X_2 system was observed at δ 8.04 (2H, d, *J* = 9.1 Hz, H-2', 6') and 6.92 (2H, d, *J* = 9.1 Hz, H-3', 5'), which could be assigned to the protons on a para-substituted phenyl ring. In the ^{13}C NMR spectrum, six aromatic methine carbons at δ 98.8 (C-6), 94.0 (C-8), 130.1 (C-2', 6'), 116.0 (d, C-3', 5'), eight aromatic quaternary carbons and a carbonyl carbon at δ 176.4 (C-4), were observed. Analysis of these data allowed to conclude the structure of **40** as the known kaempferol. The spectral data of the compound isolated in the present study match well with those reported earlier (Table 2.46).[169]

Table 2.46 Comparison of characteristic ^1H NMR (300 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6) data of kaempferol **40** with literature data (400/100 MHz, CD_3OD)[169]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1				
2		147.3		148.0
3		136.2		137.1
3-OH	9.42 (1H, brs)			
4		176.4		177.4
5		156.7		162.5
5-OH	12.48 (1H, s)			
6	6.18 (1H, d, *J* = 2.2 Hz)	98.8	6.17 (1H, d, *J* = 1.4 Hz)	99.2
7		164.5		165.6
7-OH	10.12 (1H, brs)			
8	6.43 (1H, d, *J* = 2.2 Hz)	94.0	6.38 (1H, s)	94.4
9		161.3		160.5
10		103.5		104.5
1'		122.2		123.7
2'	8.04 (1H, d, *J* = 9.1 Hz)	130.1	8.07 (1H, d, *J* = 8.7 Hz)	130.7
3'	6.92 (1H, d, *J* = 9.1 Hz)	116.0	6.89 (1H, d, *J* = 8.7 Hz)	116.3
3'-OH	10.80 (1H, brs)			
4'		159.8		158.2
5'	6.92 (1H, d, *J* = 8.8 Hz)	116.0	6.89 (1H, d, *J* = 8.7 Hz)	116.2
6'	8.04 (1H, d, J = 9.4 Hz)	130.1	8.07 (1H, d, *J* = 8.7 Hz)	130.7

Scheme 2.7 describes the complete isolation of kaempferol **40** from the aerial parts of *G. bicolor*.

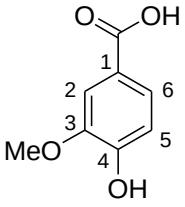
Scheme 2.7 The complete isolation of kaempferol **40** from *G. bicolor*



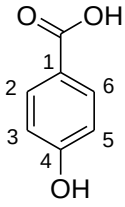
40 (6.3 mg)

2.3.5 Phenolic acids from the aerial parts of *G. bicolor*

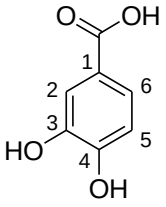
2.3.5.1 Phenolic acid compound: vanillic acid **80**, 4-hydroxybenzoic acid **79** and protocatechuic acid **157**



80



79



157

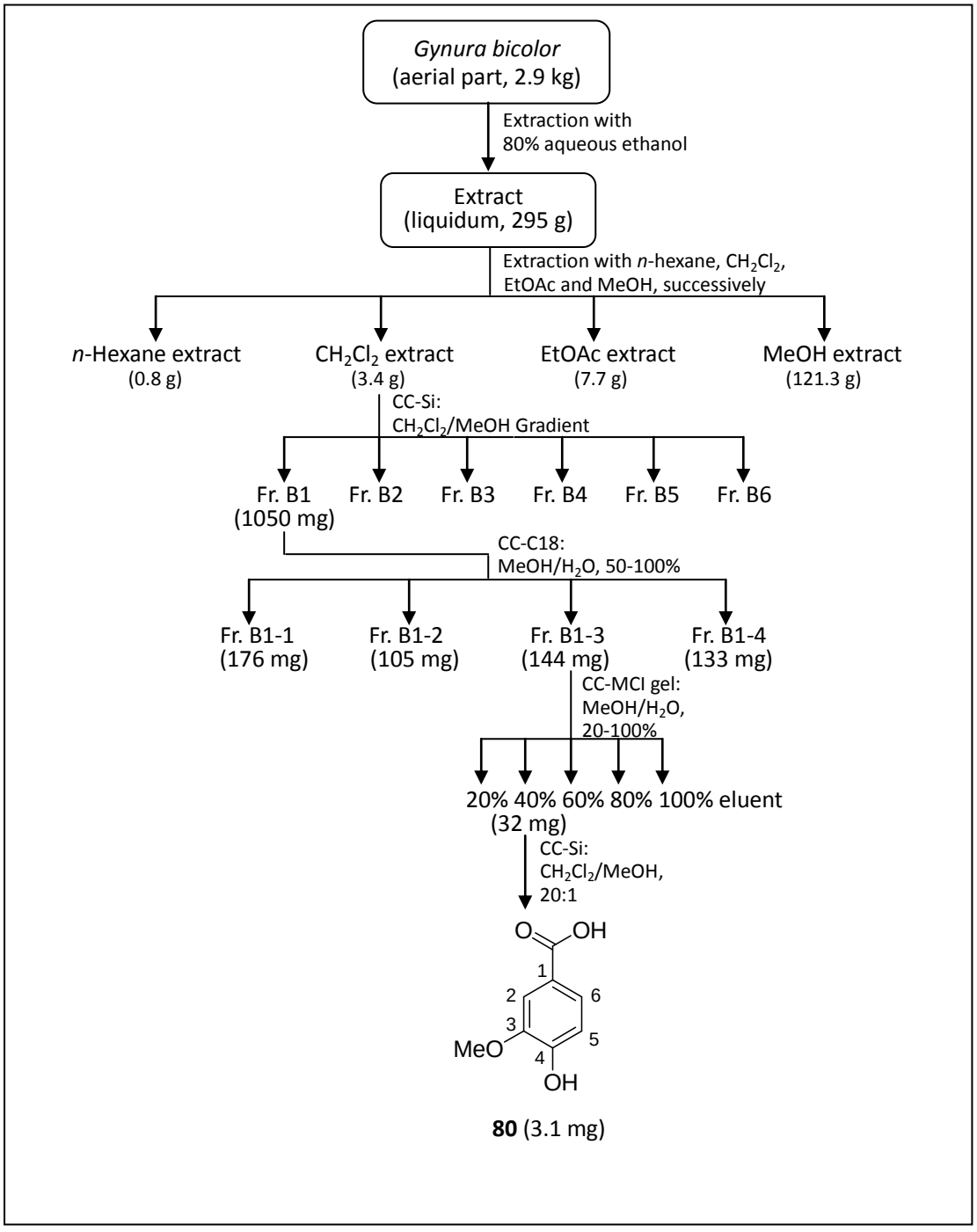
Compounds **80**, **79** and **157** were all isolated as white powder. Their ESI Mass spectram suggested their molecular formula as $C_8H_8O_4$, $C_7H_6O_3$ and $C_7H_6O_4$, respectively. In the ^{1}H NMR spectra of compounds **80** and **157**, three aromatic proton signals with an ABX coupling system were observed. In the ^{1}H NMR spectrum of compound **79**, two doublets of doublets at δ 7.77 (2H, *J* = 8.8, 3.9 Hz, H-2, 6) and δ 6.80 (2H, *J* = 8.8, 3.9 Hz, H-3, 5) were present, indicative of a 1,4-disubstituted aromatic ring. By comparison of the spectroscopic data with those reported in literature[159], the structures of **80**, **79** and **157** could be determined as vanillic acid, 4-hydroxybenzoic acid and protocatechuic acid, respectively (Table 2.47).

Table 2.47 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, DMSO-d_6) data of compounds **80**, **79** and **157** with literature data (300/75 MHz, DMSO-d_6)[159]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
Compound **80**				
1		123.0		121.7
2	7.55 (1H, d, *J* = 1.8 Hz)	115.8	7.43 (1H, s)	115.1
3		148.7		147.3
4		152.7	9.85 (1H, s)	151.1
5	6.84 (1H, d, *J* = 8.3 Hz)	113.8	6.84 (1H, d, *J* = 8.7 Hz)	112.8
6	7.56 (1H, dd, *J* = 8.3, 1.8 Hz)	125.3	7.45 (1H, d, *J* = 8.7 Hz)	123.5
COOH		170.0	12.48 (1H,s)	167.3
OCH_3	3.90 (3H, s)	56.4	3.80 (3H, s)	55.6
Compound **79**				
1		122.0		121.5
2, 6	7.77 (2H, dd, *J* = 8.8, 3.9 Hz)	132.1	7.80 (2H, dd)	131.6
3, 5	6.80 (2H, dd, *J* = 8.8, 3.9 Hz)	115.7	6.83 (2H, dd)	115.2
4		162.1		161.7
COOH		167.7	11.28 (1H, s)	167.3
Compound **157**				
1		121.8		121.8
2	7.34 (1H, s)	116.4	7.46 (1H, d, *J* = 2.0 Hz)	116.7
3		144.8		145.0
4		150.2		150.1
5	6.69 (1H, d, *J* = 8.8 Hz)	114.4	6.80 (1H, d, *J* = 8.1 Hz)	115.3
6	7.32 (1H, dd, *J* = 7.2, 1.7 Hz)	122.6	7.30 (1H, dd, *J* = 2.0, 8.1 Hz)	122.0
COOH		168.9		167.5

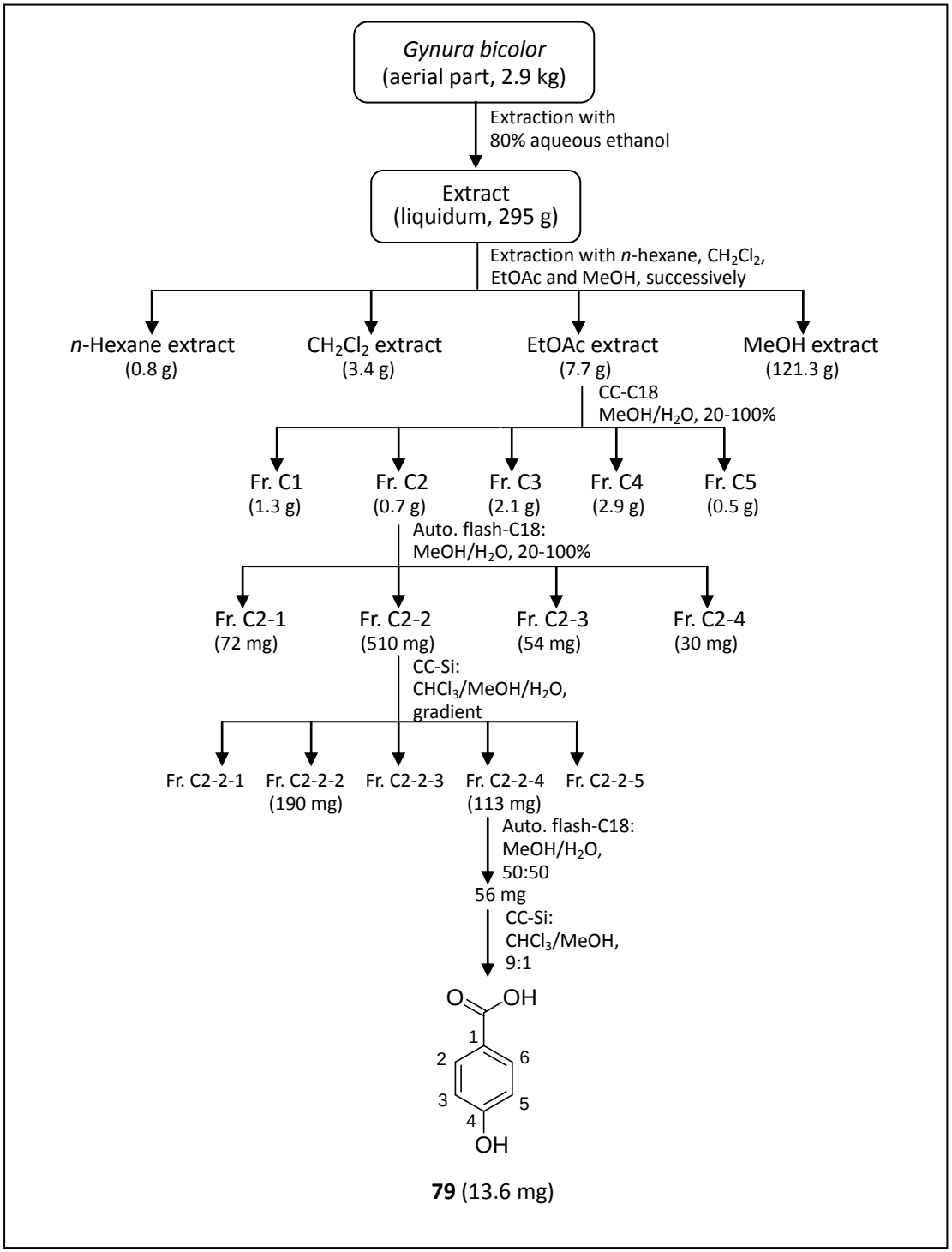
Scheme 2.8 describes the complete isolation of vanillic acid **80** from the aerial parts of *G. bicolor*.

Scheme 2.8 The complete isolation of vanillic acid **80** from *G. bicolor*



Scheme 2.9 describes the complete isolation of 4-hydroxybenzoic acid **79** from the aerial parts of *G. bicolor*.

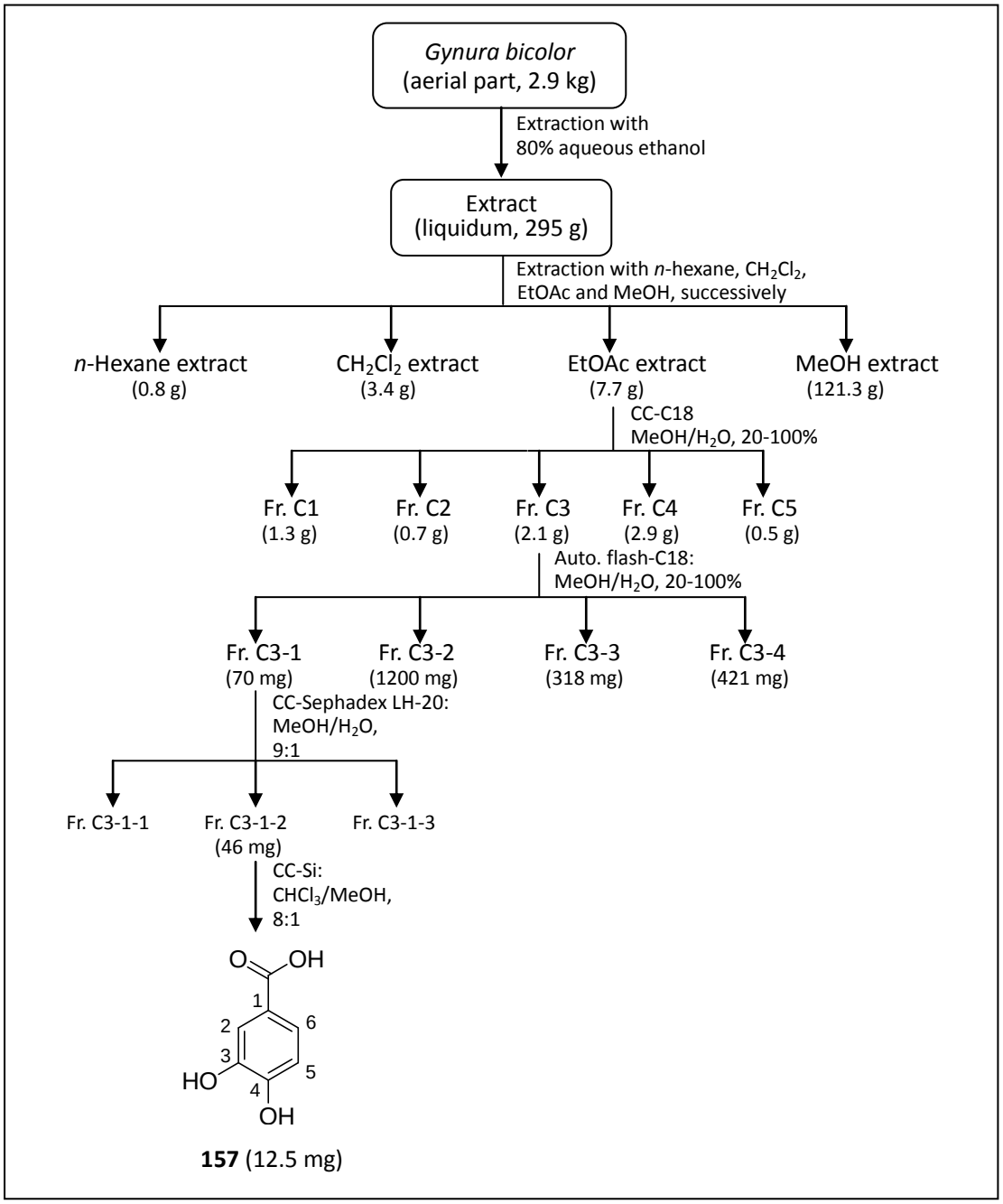
Scheme 2.9 The complete isolation of 4-hydroxybenzoic acid **79** from *G. bicolor*



79 (13.6 mg)

Scheme 2.10 describes the complete isolation of protocatechuic acid **157** from the aerial parts of *G. bicolor*.

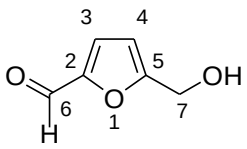
Scheme 2.10 The complete isolation of protocatechuic acid **157** from *G. bicolor*



157 (12.5 mg)

2.3.6 Other natural products from the aerial parts of *G. bicolor*

2.3.6.1 Sugar derivative: 5-(hydroxymethyl)furfural **148**



148

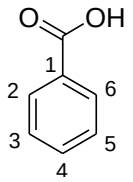
Compound **148** was obtained as a colorless oil. A quasi-molecular ion peak $[M+H]^+$ was observed with *m/z* 127 in the ESI mass spectrum suggesting that the molecular formula of **148** was $C_6H_6O_3$. In the ^1H NMR spectrum, the proton at δ 9.53 suggested an aldehyde group, two doublets at δ 6.59 and 7.48 (each 1H, d, *J* = 3.3 Hz) were attributed to β protons from a furan ring. In addition, the protons of an oxygenated methylene group were observed at δ 4.49. These data allowed to assign the structure of **148** as the known 5-(hydroxymethyl)furfural.

The spectral data of compound **148** in the present study match well with those reported earlier.[156] The comparison of characteristic NMR data of compound **148** with literature data is listed in the Table 2.48.

Table 2.48 Comparison of characteristic ^1H NMR (300 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6) data of 5-(hydroxymethyl)furfural **148** with literature data (400/100 MHz, DMSO-d_6)[156]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1				
2		152.3		152.1
3	7.48 (1H, d, *J* = 3.3 Hz)	110.3	7.42 (1H, d, *J* = 3.4 Hz)	110.0
4	6.59 (1H, d, *J* = 3.3 Hz)	125.0	6.53 (1H, d, *J* = 3.4 Hz)	124.8
5		162.8		163.2
6	9.53 (1H, s)	178.6	9.45 (1H, s)	178.3
7	4.49 (2H, s)	56.5	4.43 (2H, s)	56.0

2.3.6.2 Benzoic acid **78**



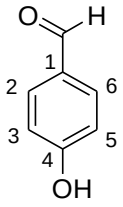
78

Compound **78** was obtained as a white powder. A quasi-molecular ion peak $[M-H]^-$ appeared at *m/z* 121 in the negative ESI mass spectrum suggesting that the molecular formula of **78** was $C_7H_6O_2$. The ^1H NMR spectrum of **78** displayed two aromatic system at ca. δ 7.49 (2H) and 7.63 (1H), one aromatic doublet at δ 8.12 (2H), respectively. The ^{13}C NMR spectrum also exhibited four aromatic signals at δ 133.9 (C-4), 130.3 (C-2 and C-6), 129.3 (C-1), 128.6 (C-3 and C-5) and one carbonyl signal at δ 171.7. Based on this, the structure of compound **78** was elucidated as benzoic acid. The spectral data of the compound isolated in the present study match well with those reported earlier (Table 2.49).[157]

Table 2.49 Comparison of characteristic ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) data of benzoic acid **78** with literature data (90/22.5 MHz, $CDCl_3$)[157]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		129.3		129.44
2, 6	8.12 (2H, d, *J* = 7.2 Hz)	130.3	8.12 (2H, m)	130.28
3, 5	7.46-7.52 (2H, m)	128.6	7.45 (2H, m)	128.49
4	7.60-7.65 (1H, m)	133.9	7.62 (1H, m)	133.83
7		171.7		172.77

2.3.6.3 4-Hydroxybenzaldehyde **149**



149

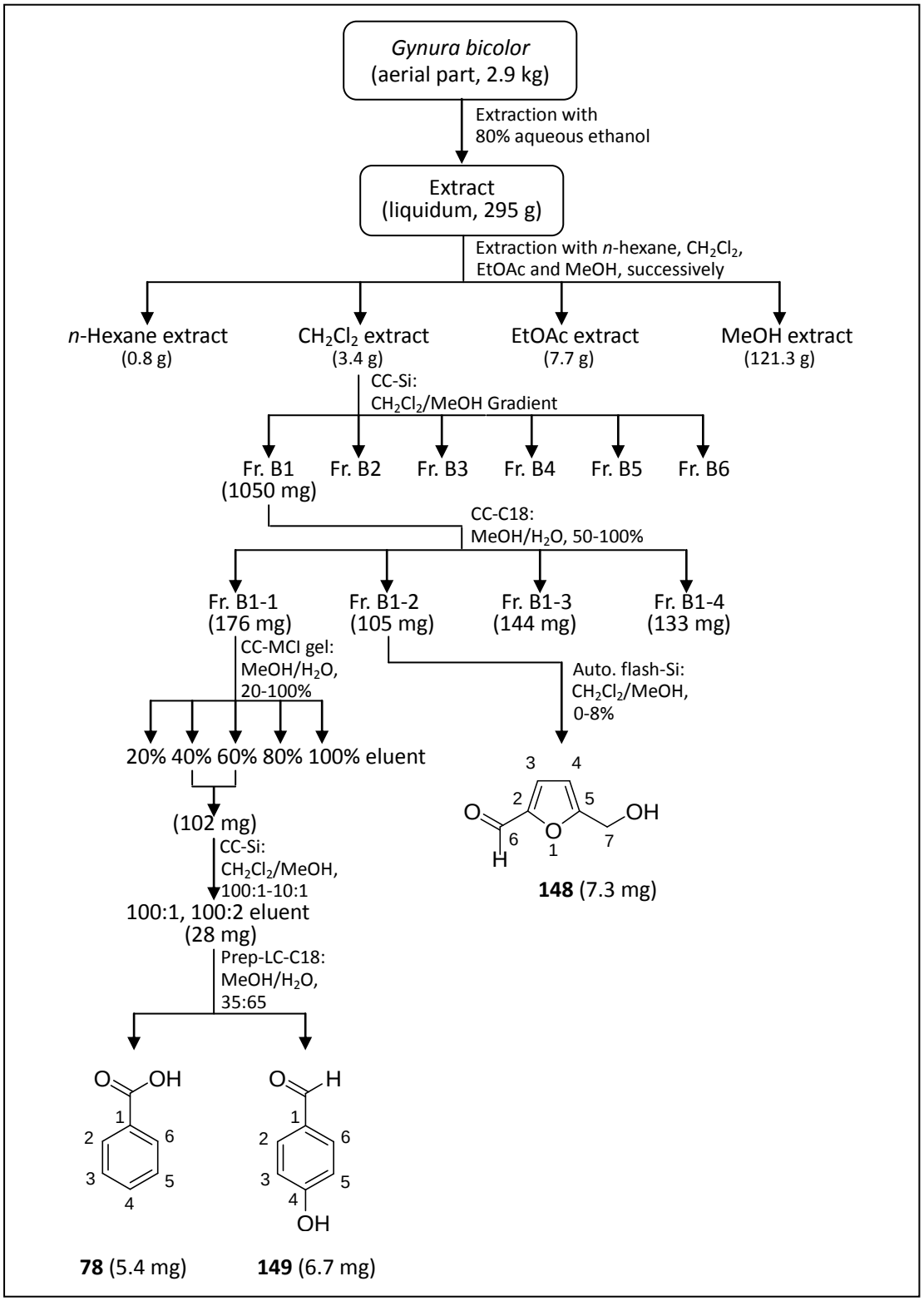
Compound **149** was obtained as a white powder. A quasi-molecular ion peak $[M-H]^-$ appeared at *m/z* 121 in the negative ESI mass spectrum suggesting that the molecular formula of **149** was $C_7H_6O_2$. The ^{1}H NMR spectra of compound **149** showed the characteristic pattern of a 1,4-disubstituted benzene ring with two doublets at δ 7.76 (2H, d, *J* = 8.5 Hz, H-2, 6) and 6.89 (2H, d, *J* = 8.5 Hz, H-3, 5). At the same time, an aldehyde proton was observed at 9.74 as well. Moreover, the ^{13}C NMR data were consistent with 4-hydroxybenzaldehyde (Table 2.50).[158]

Table 2.50 Comparison of characteristic ^{1}H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of 4-hydroxybenzaldehyde **149** with literature data (400/100 MHz, DMSO-d_6)[158]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		130.1		128.5
2, 6	7.76 (2H, d, *J* = 8.5 Hz)	132.5	7.79 (2H, d)	132.0
3, 5	6.89 (2H, d, *J* = 8.5 Hz)	116.0	6.97 (2H, d)	115.8
4		161.4		163.3
7	9.74 (1H, s)	190.9	9.82 (1H, s)	190.8

Scheme 2.11 describes the complete isolation of compounds **148**, **78**, **149** from the aerial parts of *G. bicolor*.

Scheme 2.11 The complete isolation of compounds **148**, **78**, **149** from *G. bicolor*



Chapter 3

STUDY OF THE CHEMICAL CONSTITUENTS OF THE AERIAL PARTS OF *GYNURA DIVARICATA*

3.1 Introduction

Gynura divaricata (Linnaeus) Candolle is a herb with erect stems, glabrous or shortly pubescent, about 30-60 cm high and purplish; their leaves are ovate or half-moon-shaped, dentate auricle at base; blade adaxially purplish while abaxially green, which is reverse compared with *Gynura bicolor* (Figure 3.1). Capitula are numerous, peduncles are 1-15 cm; involucres campanulate; florets are orange-yellow; corolla slightly exceeding involucres, 11-15 mm; tube 9-10 mm; lobes are oblong-ovate, while anthers are obtuse. Style branches are slender. Achenes are brown, cylindric, 10-ribbed. Pappus white and silky. The flower time is from August to October.[12]

Figure 3.1. *Gynura divaricata* (Linnaeus) Candolle

Gynura divaricata is a traditional Chinese medicine, which can be used for the treatment of bronchitis, pulmonary tuberculosis, pertussis, sore eye, toothache and rheumatic arthralgia.[174] *G. divaricata* is also known as an antidiabetic plant. It is used in folk recipes for the treatment of diabetes mellitus in Jiangsu, Zhejiang and Sichuan province in south China. In our investigation of natural medicines used in the traditional Chinese medical system for treatment of diabetes,[52,151] a tea made from the fresh leaves of *G. divaricata* was found to have excellent hypoglycemia effects. Our previous pharmacological tests proved that the ethyl acetate and *n*-butanol extracts of the aerial parts of the plant had significant effects on lowering blood glucose level in normal and alloxan diabetic mice.[151] However, there are few reported phytochemical investigations of this species, despite the possible relationship between the hypoglycemic activity and the natural products from this species. One literature disclosure reported two pyrrolizidine alkaloids from this species[22] and other researchers found cerebrosides[41,42,43] and flavonoids[31] from this plant. Therefore, this further research on the isolation and identification of the active chemical constituents from *G. divaricata* was conducted. The biological testing of the isolated compounds on potential hypoglycemic activity, en route towards the development of natural products for the treatment of diabetes mellitus, was planned to be studied as well.

3.2 Experimental

3.2.1 Plant materials

The aerial parts of *G. divaricata* for chemical constituent investigation were collected in June 2010 in Nanjing Botanical Garden Mem. Sun Yat-sen, in the south of the Zijin Mountain, Nanjing, China. The plant was identified by Professor Guo Rong-lin at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. A voucher specimen (No. 510310-2) was deposited in the herbarium, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences.

3.2.2 Instrumentation

Silica gel 60 (0.015-0.040 mm for column chromatography; Merck) was used as normal phase, whereas LiChroprep RP-18 (40-63 μm particle size; Merck) was used as reversed phase column material. MCI gel CHP20P (75-150μm, 400-600 Å mean pore size; Mitsubishi Chemical Corp.) and Sephadex LH-20 (GE healthcare) were used for column chromatography as well. TLC analyses were carried out on silica gel plates (KG60-F254, Merck). The melting point was measured with an X-6 micro-melting point apparatus (Beijing Tech). ^{1}H and ^{13}C NMR spectra were obtained on a JEOL 300 spectrometer. The HPLC instrument consisted of an Agilent (Waldbronn, Germany) model 1100 liquid chromatograph with diode array detector. The HPLC-MS consisted of Agilent LC/MSD 1100 series, and the prep-LC consisted of Agilent 1100 serious equipped with VWD detector and automatic fraction collector. Automatic flash chromatography was performed by The Reveleris Flash System from GRACE, United States.

3.2.3 Extraction and purification

3.2.3.1 Extraction

Dry aerial parts of *G. divaricata* (7.5 kg) were cut into small pieces and extracted with 80% aqueous ethanol at 70 °C twice to afford 900 g of crude extract after evaporation in vacuo of the solvent. The crude extract was suspended in water (4.5 L), then liquid-liquid partitioned successively by *n*-hexane, EtOAc and *n*-butanol, respectively. The extracts were filtered and concentrated by evaporation under reduced pressure with a rotavapor at 40 °C to afford a dark green *n*-hexane residue, a dark brown EtOAc residue (47.5 g, 6.33 % yield), and a dark brown *n*-butanol residue.

3.2.3.2 Study of the ethyl acetate extract of the aerial parts of *G. divaricata*

The EtOAc extract (47.5 g) was adsorbed on 50 g of silica gel and further fractionated by column chromatography over silica gel, eluted with a gradient solvent system (*n*-hexane/EtOAc/acetone, Table 3.1). The fractions were collected in 100 mL tubes and after monitoring by TLC under UV, a total of seven different combined fractions (Fr. D1-7) were obtained (Table 3.2).

Table 3.1 Programme for fractionation of the EtOAc extract of the aerial parts of *G. divaricata* by column chromatography

Mobile phase	Gradient elution	Solvent volume (mL)
n-hexane	100	500
n-hexane/EtOAc	100:10	1100
n-hexane/EtOAc	100:25	2500
n-hexane/EtOAc	50:50	3000
n-hexane/EtOAc	50:100	4500
EtOAc	100	2400
EtOAc/acetone	100:25	2500
EtOAc/acetone	100:50	1500
EtOAc/acetone	50:50	1000
acetone (clean up)	100	1000

Table 3.2 Column chromatography fractionation of the EtOAc extract of *G. divaricata*

Fraction	Weight (mg)
Fr. D1	0.8 g
Fr. D2	2.1 g
Fr. D3	7.0 g
Fr. D4	7.6 g
Fr. D5	11.3 g
Fr. D6	8.1 g
Fr. D7	---

3.2.3.2.1 Isolation of succinic acid **94**

Fr. D3 (7.0 g) was adsorbed on silica gel (12 g) and chromatographed over a normal phase column. Upon elution by a gradient of petroleum ether/EtOAc/acetone (Table 3.3) and monitoring by TLC, five combined fractions (Fr. D3-1~5) were obtained (Table 3.4).

Table 3.3 Programme for separation of fraction Fr. D3 of the aerial parts of *G. divaricata* by column chromatography

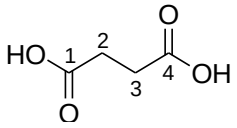
Mobile phase	Gradient elution	Solvent volume (mL)
PE/EtOAc/acetone	100:5:5	1000
PE/EtOAc/acetone	100:8:8	1000
PE/EtOAc/acetone	100:10:10	1000
PE/EtOAc/acetone	100:20:20	2000
PE/EtOAc/acetone	100:25:25	1500
PE/EtOAc/acetone	100:50:50	1500
acetone (clean up)	100	800

Table 3.4 Column chromatography fractionation of Fr. D3 of *G. divaricata*

Fraction	Weight (mg)
Fr. D3-1	220
Fr. D3-2	540
Fr. D3-3	1600
Fr. D3-4	3500
Fr. D3-5	800

Fr. D3-3 (1.6 g) was dissolved in a small amount of hot EtOAc to fully dissolve the mixture and then filtered to obtain a clear solution. After cooling overnight, it resulted in compound **94** (1.1 g) which crystallized from the solvent. The solid crystals were collected by filtration as colorless needles.

By comparison of the ^{1}H, ^{13}C NMR and ESI-MS spectral data with those reported,[175] the compound **94** was identified as succinic acid.



94

Succinic acid 94: colorless needles; m.p. 176-177 °C; ESI-MS m/z: 117 $[M-H]^-$; ^{1}H NMR δ ppm (D_2O, 300 MHz): 2.57 (4H, s, H-2 and H-3); ^{13}C NMR δ ppm (D_2O, 75 MHz): 174.1 (C-1 and C-4), 29.3 (C-2 and C-3).

3.2.3.2.2 Isolation of salicylic acid **160** and ethyl methyl succinate **161**

Fr. D3-4 (3.5 g) was subjected to column chromatography over silica gel eluted by a gradient of petroleum ether/EtOAc/acetone for further separation (Table 3.5). After monitoring by TLC, four combined fractions (Fr. D3-4-1~4) were obtained (Table 3.6).

Table 3.5 Programme for separation of fraction Fr. D3-4 of *G. divaricata* by column chromatography

Mobile phase	Gradient elution	Solvent volume (mL)
PE/EtOAc/acetone	10:1:1	1800
PE/EtOAc/acetone	8:1:1	1500
PE/EtOAc/acetone	5:1:1	1400
PE/EtOAc/acetone	4:1:1	1200
PE/EtOAc/acetone	2:1:1	1200

Table 3.6 Column chromatography fractionation of Fr. D3 of *G. divaricata*

Fraction	Weight (mg)
Fr. D3-4-1	150
Fr. D3-4-2	1300
Fr. D3-4-3	800
Fr. D3-4-4	500

Fr. D3-4-2 (1300 mg) was fractionated with an automatic flash chromatography system on a normal phase column eluted by a gradient of CH_2Cl_2 and methanol (0-9 %). The automatic flash chromatography conditions were (Table 3.7 and 3.8):

Table 3.7 Automatic flash chromatography conditions of Fr. D3-4-2 of *G. divaricata*

Run conditions	
Cartridge:	Reveleris 40 g
Solvent A:	CH_2Cl_2
Solvent B:	Methanol
Flow rate:	50 mL/min
UV1 wavelength:	254 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 3.8 Automatic flash chromatography gradient method of Fr. D3-4-2 of *G. divaricata*

Gradient method		
Step	Time (min.)	%B
1	0	0
2	90	9

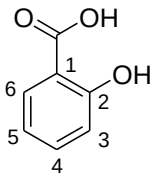
The eluates from 3.6-5.1 and 10.2-12.4 min were collected separately, concentrated by evaporation under reduced pressure with a rotavapor and finally dried under high vacuum. In this way, two pure compounds **160** (3.0 mg) and **161** (6.7 mg) were yielded.

Besides the above two collected eluates, the others were collected as well and concentrated by evaporation under reduced pressure with a rotavapor, after monitoring by TLC under UV, four combined fractions (Fr. D3-4-2-1~4) were obtained (Table 3.9).

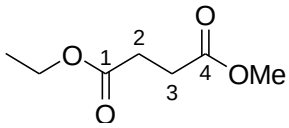
Table 3.9 Column chromatography fractionation of Fr. D3-4-2 of *G. divaricata*

Fraction	Weight (mg)
Fr. D3-4-2-1	103
Fr. D3-4-2-2	52
Fr. D3-4-2-3	89
Fr. D3-4-2-4	170

Analysis of their spectral data, together with comparison with data available in the literature,[175,176,177] allowed to elucidate the structure of compounds **160** and **161** and to identify them as salicylic acid and ethyl methyl succinate, respectively.



160



161

Salicylic acid 160: white powder; m.p. 154-155 °C; ESI-MS: m/z 137 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.85 (1H, dd, *J* = 8.2, 1.7 Hz, H-6), 7.46 (1H, ddd, *J* = 8.3, 7.2, 1.7 Hz, H-4), 6.91 (1H, ddd, *J* = 8.2, 7.2, 1.1 Hz, H-5), 6.87 (1H, dd, *J* = 8.3, 1.1 Hz, H-3); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 173.5 (-COOH), 163.2 (C-2), 136.6 (C-4), 131.5 (C-6), 120.1 (C-5), 118.1 (C-3), 113.9 (C-1).

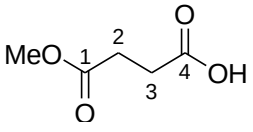
Ethyl methyl succinate 161: colorless liquid; ^{1}H NMR δ ppm ($CDCl_3$, 300 MHz): 4.16 (2H, q, *J* = 7.2 Hz, -OC<u>H</u>$_2$), 3.71 (3H, s, OC<u>H</u>$_3$), 2.60-2.73 (4H, m, 2 × -COC<u>H</u>$_2$), 1.27 (3H, t, *J* = 7.2 Hz, -CH$_2$C<u>H</u>$_3$); ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 172.8 (C-4), 172.3 (C-1), 61.0 (O<u>C</u>H$_2$), 52.1 (O<u>C</u>H$_3$), 28.9 (C-2 and C-3), 14.2 (-CH$_2$<u>C</u>H$_3$).

3.2.3.2.3 Isolation of methyl succinate **162** and ethyl succinate **163**

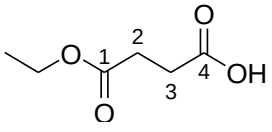
Fr. D3-4-2-4 (170 mg) was subjected to column chromatography over Sephadex LH-20 gel eluted with methanol for further separation. The collected fractions were combined according to their chromatographic behavior on the TLC using CH_2Cl_2/MeOH (10:1) as eluent. The fraction with Rf 0.34 (30.2 mg) was additionally purified by prep-HPLC. The prep-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvent isocratic 28% MeCN in water; flow rate 6 mL/min; detection UV 210 nm; room temperature; injection volume 150 μL per time; running time 20 mins per injection; automatic fraction collector mode peak-based.

The eluent from 8.7 to 9.9 min and 10.0 to 11.2 min were collected, respectively. Solvent removal by evaporation under reduced pressure yielded two pure compounds (**162**, 4.3 mg and **163**, 3.2 mg).

By comparison of their ^{1}H, ^{13}C NMR and ESI-MS spectral data with those reported,[175] the two compounds were identified as methyl succinate **162** and ethyl succinate **163**, respectively.



162



163

Methyl succinate 162: white powder; m.p. 54-55 °C; ESI-MS m/z: 131 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 3.57 (3H, s, -OC<u>H</u>$_3$), 2.49-2.51 (4H, m, H-2 and H-3); ^{13}C NMR δ ppm (75 MHz, MeOH-d_4): 174.8 (C-4), 173.5 (C-1), 50.9 (O<u>C</u>H$_3$), 28.5 (C-2 and C-3).

Ethyl succinate 163: white powder; m.p. 53-54 °C; ESI-MS m/z: 145 $[M-H]^-$; ^{1}H NMR δ ppm ($CDCl_3$, 300 MHz): 4.16 (2H, q, *J* = 6.9 Hz, -OC<u>H</u>$_2$), 2.38-2.69 (4H, m, H-2 and H-3), 1.26 (3H, t, *J* = 6.9 Hz, -C<u>H</u>$_3$); ^{13}C NMR δ ppm ($CDCl_3$, 75 MHz): 176.4 (C-1 and C-4), 60.9 (O<u>C</u>H$_2$), 28.5 (C-2 and C-3), 14.1 (-<u>C</u>H$_3$).

3.2.3.2.4 Isolation of isovanillic acid **164**

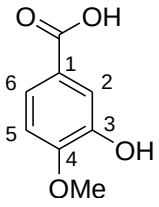
Fr. D3-4-4 (500 mg) was fractionated with column chromatography over silica gel eluted by CH_2Cl_2/methanol (10:1). The collected fractions were concentrated by evaporation under reduced pressure with a rotavapor and after monitoring by TLC with UV detection, three combined fractions (Fr. D3-4-4-1~3) were obtained (Table 3.10).

Table 3.10 Column chromatography fractionation of Fr. D3-4-4 of *G. divaricata*

Fraction	Weight (mg)
Fr. D3-4-4-1	62
Fr. D3-4-4-2	182
Fr. D3-4-4-3	55

Fr. D3-4-4-2 (182 mg) was dissolved in a small amount of hot CH_2Cl_2/acetone to fully dissolve the mixture. After cooling overnight, this resulted in compound **164** (23.1 mg) which crystallized from the solvent.

Analysis of the spectral data, together with comparison with data available in the literature,[178] allowed to elucidate the structure of compound **164** and to identify it as isovanillic acid.



164

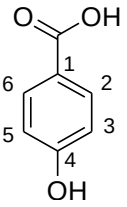
Isovanillic acid 164: white powder; m.p. 248-249 °C; ESI-MS m/z: 167 $[M-H]^-$; ^{1}H NMR δ ppm (300 MHz, MeOH-d_4): 7.48 (1H, dd, *J* = 8.7, 1.7, H-6), 7.47 (1H, d, *J* = 1.7 Hz, H-2), 6.75 (1H, d, *J* = 8.7 Hz, H-5), 3.81 (3H, s, -OC<u>H</u>$_3$); ^{13}C NMR δ ppm (75 MHz, MeOH-d_4): 168.8 (-<u>C</u>OOH), 151.3 (C-4), 147.3 (C-3), 123.9 (C-6), 121.8 (C-1), 114.5 (C-2), 112.4 (C-5), 55.0 (-O<u>C</u>H$_3$).

3.2.3.2.5 Isolation of 4-hydroxybenzoic acid **79** and *p*-coumaric acid **165**

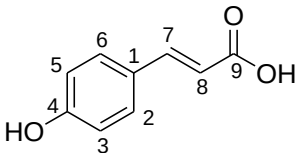
Fr. D3-4-4-3 (55 mg) was additionally purified by prep-HPLC. The prep-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvent isocratic 26% MeCN in water; flow rate 6 mL/min; detection UV 254 nm; room temperature; injection volume 150 μL per time; running time 15 mins per injection; automatic fraction collector mode peak-based.

The eluents from 9.3-11.1 min and 11.4-12.7 min were collected, respectively. Solvent removal by evaporation under reduced pressure yielded two pure compounds (**79**, 5.6 mg; **165**, 4.8 mg).

By comparison of their ^{1}H, ^{13}C NMR and ESI-MS spectral data with those reported,[179] the two compounds were identified as 4-hydroxybenzoic acid **79** and *p*-coumaric acid **165**, respectively.



79



165

4-Hydroxybenzoic acid 79: white powder; m.p. 211-212 °C; ESI-MS m/z: 137 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.77 (2H, d, *J* = 8.3 Hz, H-2 and H-6), 6.71 (2H, d, *J* = 8.3 Hz, H-3 and H-5); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 169.0 (-COOH), 161.9 (C-4), 131.7 (C-2 and C-6), 121.7 (C-1), 114.7 (C-3 and C-5).

***p*-Coumaric acid 165**: white powder; m.p. 209-210 °C; ESI-MS m/z: 163 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.52 (1H, d, *J* = 15.9 Hz, H-7), 7.37 (2H, d, *J* = 8.8 Hz, H-2, 6), 6.73 (2H, d, *J* = 8.8 Hz, H-3, 5), 6.20 (1H, d, *J* = 15.9, H-8); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 169.7 (COOH), 159.9 (C-4), 145.3 (C-7), 129.8 (C-2 and C-6), 125.9 (C-1), 115.5 (C-3 and C-5), 114.3 (C-8).

3.2.3.2.6 Isolation of esculetin **166**

Fr. D3-5 (800 mg) was further separated by column chromatography over silica eluted by a gradient of CH_2Cl_2 and methanol (Table 3.11) resulting in three fractions (Fr. D3-5-1~3) (Table 3.12). Fr. D3-5-2 (177 mg) was recrystallized in acetone to yield compound **166** (12.7 mg).

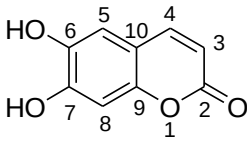
Table 3.11 Programme for separation of Fr. D3-5 of *G. divaricata*

Mobile phase	Gradient elution	Solvent volume (mL)
CH_2Cl_2	100	300
CH_2Cl_2/MeOH	100:2	1530
CH_2Cl_2/MeOH	100:5	525
CH_2Cl_2/MeOH	100:10	550
CH_2Cl_2/MeOH	100:20	600

Table 3.12 Column chromatography fractionation of Fr. D3-5 of *G. divaricata*

Fraction	Weight (mg)
Fr. D3-5-1	153
Fr. D3-5-2	177
Fr. D3-5-3	86

Analysis of the spectral data, together with comparison with data available in the literature,[180] allowed to elucidate the structure of compound **166** as esculetin.



166

Esculetin 166: yellow crystals; m.p. 266-267 °C; ESI-MS m/z: 177 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.70 (1H, d, *J* = 9.9 Hz, H-4), 6.85 (1H, s, H-5), 6.67 (1H, s, H-8), 6.09 (1H, d, *J* = 9.9 Hz, H-3); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 163.0 (C-2), 150.7 (C-7), 149.1 (C-9), 144.7 (C-4), 143.2 (C-6), 111.7 (C-5), 111.5 (C-3), 111.2 (C-10), 102.3 (C-8).

3.2.3.2.7 Isolation of rutin **38**

Fr. D4 (7.6 g) was subjected to column chromatography over MCI gel, eluted with water containing increasing concentrations of MeOH (Table 3.13). After monitoring by TLC, a total of five different combined fractions (Fr. D4-1~5) were obtained (Table 3.14).

Table 3.13 Programme for fractionation of Fr. D4 of *G. divaricata* by column chromatography

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	800
MeOH/H_2O	40:60	2000
MeOH/H_2O	60:40	2000
MeOH/H_2O	80:20	1500
MeOH (clean up)	100	500

Table 3.14 Column chromatography fractionation of Fr. D4 of *G. divaricata*

Fraction	Weight (g)
Fr. D4-2	1.3
Fr. D4-3	0.9
Fr. D4-4	0.8
Fr. D4-5	1.1

Fr. D4-2 (1.3 g) was further fractionated with an automatic flash chromatography system on a reversed-phase column (C-18, 40 g) eluted by a gradient of water and methanol (20-100%). The automatic flash chromatography conditions were (Table 3.15 and 3.16):

Table 3.15 Automatic flash chromatography conditions of Fr. D4-2 of *G. divaricata*

Run conditions	
Cartridge:	Reveleris 40 g C18
Solvent A:	Water
Solvent B:	Methanol
Flow rate:	25 mL/min
UV1 wavelength:	254 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 3.16 Automatic flash chromatography gradient method of Fr. D4-2 of *G. divaricata*

Gradient method		
Step	Time (min.)	%B
1	0	20
2	60	100

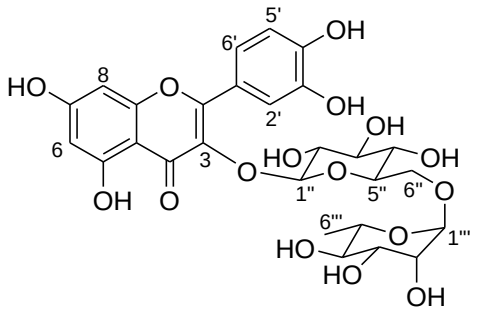
Upon monitoring by TLC under UV, four different fractions (Fr. D4-2-1~4) were obtained (Table 3.17), after concentration by evaporation under reduced pressure with a rotavapor.

Table 3.17 Column chromatography fractionation of Fr. D4-2 of *G. divaricata*

Fraction	Weight (mg)
Fr. D4-2-1	315
Fr. D4-2-2	208
Fr. D4-2-3	136
Fr. D4-2-4	46

Fr. D4-2-2 (208 mg) was further subjected to column chromatography over silica gel eluted by a solvent system consisting of $CHCl_3$/MeOH (4:1). The collected fraction was finally purified by column chromatography over Sephadex LH-20 eluted with methanol to yield a pure compound (**38**, 8.9 mg).

Analysis of its spectral data, together with comparison with data available in the literature,[181] allowed to elucidate the structure of compound **38** as quercetin 3-O-rutinoside (rutin).



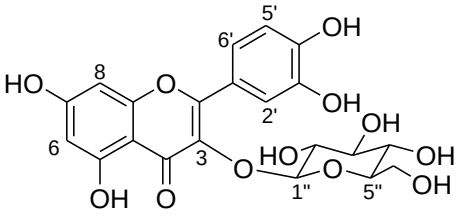
38

Rutin 38: yellow powder; m.p. 197-198 °C; ESI-MS m/z: 609 $[M-H]^-$; ^{1}H NMR δ ppm (300 MHz, MeOH-d4): 7.57 (1H, d, *J* = 2.2 Hz, H-2′), 7.54 (1H, dd, *J* = 8.7, 2.2 Hz, H-6′), 6.78 (1H, d, *J* = 8.7 Hz, H-5′), 6.31 (1H, s, H-8), 6.12 (1H, s, H-6), 5.01 (1H, d, *J* = 7.2 Hz, H-1″), 4.42 (1H, brs), 3.26-3.73 (11H, m, H-sugar), 1.02 (3H, d, *J* = 5.4 Hz, -C$\underline{H}_3$); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 178.1 (C-4), 164.7 (C-7), 161.7 (C-5), 158.0 (C-2), 157.2 (C-9), 148.5 (C-4′), 144.5 (C-3′), 134.3 (C-3), 122.2 (C-6′), 121.8 (C-1′), 116.4 (C-5′), 114.7 (C-2′), 104.3 (C-10), 103.4 (C-1″), 101.1 (C-1‴), 98.6 (C-6), 93.5 (C-8), 76.9 (C-5″), 75.9 (C-3″), 74.4 (C-2″), 72.6 (C-4‴), 70.9 (C-4″), 70.8 (C-2‴), 70.1 (C-3‴), 68.4 (C-5‴), 67.2 (C-6″), 16.6 (C$\underline{H}_3$).

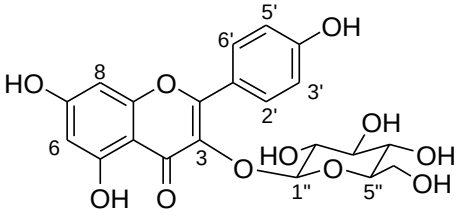
3.2.3.2.8 Isolation of quercetin 3-O-β-D-glucoside **36** and kaempferol 3-O-β-D-glucoside **41**

Fr. D4-2-3 (136 mg) was further subjected to column chromatography over silica gel eluted by a solvent system consisted of $CHCl_3$/MeOH (6:1). Based on TLC analysis, the combined eluent (37 mg) was finally purified by prep-HPLC on a RP-C18 column to afford two pure products. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); elution, isocratic mode with MeOH/H_2O (55:45); flow rate, 6 mL/min; detection, UV 254 nm; room temperature; injection volume, 150 μL per time; running time, 12.5 mins per injection; automatic fraction collector mode peak-based. The sample was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. The eluates from 8.6-9.7 and 11.0-12.2 min were collected, dried under high vacuum to afford compound **36** (2.5 mg) and **41** (2.8 mg), respectively.

Analysis of their spectral data, together with comparison with data available in the literatures,[182,183] allowed to elucidate the structure of the compound **36** and **41** as quercetin 3-O-β-D-glucoside and kaempferol 3-O-β-D-glucoside, respectively.



36



41

Quercetin 3-O-β-D-glucoside 36: yellow powder; m.p. 217-218 °C; ESI-MS m/z: 463 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.60 (1H, d, *J* = 2.2 Hz, H-2′), 7.49 (1H, dd, *J* = 8.8, 2.2 Hz, H-6′), 6.77 (1H, d, *J* = 8.8 Hz, H-5′), 6.27 (1H, d, *J* = 1.9 Hz, H-8), 6.08 (1H, d, *J* = 1.9 Hz, H-6), 5.13 (1H, d, *J* = 7.2 Hz, H-1″), 3.30-3.64 (6H, m, H-2″-6″); ^{13}C NMR δ ppm (MeOH-d_4, 100 MHz): 179.5 (C-4), 166.5 (C-7), 163.1 (C-5), 159.0 (C-9), 158.5 (C-2), 149.9 (C-4′), 147.0 (C-3′), 135.6 (C-3), 123.2 (C-1′), 123.1 (C-6′), 117.5 (C-5′), 116.0 (C-2′), 105.6 (C-10), 104.3 (C-1″), 100.0 (C-6), 94.8 (C-8), 78.4 (C-5″), 78.1 (C-3″), 75.7 (C-2″), 71.2 (C-4″), 62.6 (C-6″).

Kaempferol 3-O-β-D-glucoside 41: yellow powder; m.p. 207-208 °C; ESI-MS m/z: 447 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.96 (2H, d, *J* = 8.8 Hz, H-2′, 6′), 6.79 (2H, d, *J* = 8.8 Hz, H-3′, 5′), 6.25 (1H, d, *J* = 1.8 Hz, H-8), 6.07 (1H, d, *J* = 1.8 Hz, H-6), 5.12 (1H, d, *J* = 6.9 Hz, H-1″), 3.30-3.62 (6H, m, H-2″-6″).

3.2.3.2.9 Isolation of quercetin **35** and kaempferol **40**

Fr. D4-3 (0.9 g) was further subjected to column chromatography over silica gel eluted by a gradient of CH_2Cl_2 and methanol (100:5-8:1) (Table 3.18). Five fractions (Table 3.19) were obtained and two of them were combined for subsequent purification.

Table 3.18 Programme for separation of Fr. D4-3 of *G. divaricata*

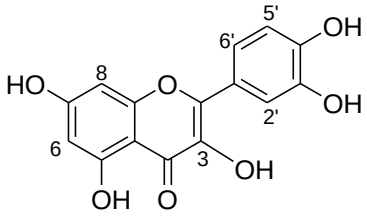
Mobile phase	Gradient elution	Solvent volume (mL)
CH_2Cl_2/MeOH	100:5	550
CH_2Cl_2/MeOH	100:10	1100
CH_2Cl_2/MeOH	90:10	500
CH_2Cl_2/MeOH	80:10	450

Table 3.19 Column chromatography fractionation of Fr. D4-3 of *G. divaricata*

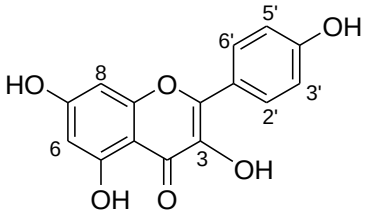
Fraction	Weight (mg)
Fr. D4-3-1	164
Fr. D4-3-2	76
Fr. D4-3-3	65
Fr. D4-3-4	51
Fr. D4-3-5	29

Fr. D4-3-2 and Fr. D4-3-3 were combined (141 mg) and chromatographed on a Sephadex LH-20 column eluted with chloroform-methanol (1:1) to afford two pure compounds **35** (10.2 mg) and **40** (8.8 mg), respectively.

Analysis of their spectral data, together with comparison with data available in the literatures,[182,183] allowed to elucidate the structure of compound **35**, **40** as quercetin and kaempferol, respectively.



35



40

Quercetin 35: yellow powder; m.p. 310-311 °C; ESI-MS m/z: 301 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.63 (1H, d, *J* = 2.1 Hz, H-2′), 7.53 (1H, dd, *J* = 8.1, 2.1 Hz, H-6′), 6.78 (1H, d, *J* = 8.1 Hz, H-5′), 6.29 (1H, d, *J* = 1.8 Hz, H-8), 6.08 (1H, d, *J* = 1.8 Hz, H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 176.0 (C-4), 164.2 (C-7), 161.2 (C-9), 156.9 (C-5), 147.4 (C-4′), 146.7 (C-2), 144.9 (C-3′), 135.9 (C-3), 122.8 (C-1′), 120.3 (C-6′), 114.9 (C-5′), 114.7 (C-2′), 103.2 (C-10), 97.9 (C-6), 93.1 (C-8).

Kaempferol 40: yellow powder; m.p. 275-276 °C; ESI-MS m/z: 287 $[M+H]^+$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.99 (2H, d, *J* = 9.1 Hz, H-2′, 6′), 6.81 (2H, d, *J* = 9.1 Hz, H-3′, 5′), 6.30 (1H, d, *J* = 2.1 Hz, H-8), 6.08 (1H, d, *J* = 2.1 Hz, H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 175.8 (C-4), 164.3 (C-7), 161.2 (C-9), 159.1 (C-4′), 156.9 (C-5), 146.6 (C-2), 135.9 (C-3), 129.4 (C-2′ and C-6′), 122.2 (C-1′), 115.0 (C-3′ and C-5′), 103.2 (C-10), 97.9 (C-6), 93.1 (C-8).

3.2.3.2.10 Isolation of 3,4-dicaffeoylquinic acid **167**, 3,5-dicaffeoylquinic acid **72**, 4,5-dicaffeoylquinic acid **73**, methyl 3,4-dicaffeoylquinate **168**, methyl 3,5-dicaffeoylquinate **169** and methyl 4,5-dicaffeoylquinate **170**

Fr. D5 (11.3 g) was subjected to column chromatography over MCI gel, eluted with water containing increasing concentrations of MeOH (Table 3.20). After monitoring by TLC, a total of four different combined fractions (Fr. D5-1~4) were obtained (Table 3.21).

Table 3.20 Programme for fractionation of Fr. D5 of the aerial parts of *G. divaricata* by column chromatography

Mobile phase	Gradient elution	Solvent volume (mL)
MeOH/H_2O	20:80	1200
MeOH/H_2O	40:60	2500
MeOH/H_2O	60:40	3000
MeOH/H_2O	80:20	2500
MeOH (clean up)	100	800

Table 3.21 Column chromatography fractionation of Fr. D5 of *G. divaricata*

Fraction	Weight (g)
Fr. D5-1	2.4
Fr. D5-2	1.3
Fr. D5-3	6.2
Fr. D5-4	0.8

Fr. D5-3 (6.2 g) was divided into four equal parts and each part was fractionated with an automatic flash chromatography system on a reversed-phase column (C-18, 40 g) eluted by a gradient of water and methanol (20-100%). The automatic flash chromatography conditions were (Table 3.22 and 3.23):

Table 3.22 Automatic flash chromatography conditions of Fr. D5-3 of *G. divaricata*

Run conditions	
Cartridge:	Reveleris 40 g C18
Solvent A:	Water
Solvent B:	Methanol
Flow rate:	25 mL/min
UV1 wavelength:	254 nm
UV2 wavelength:	280 nm
ELSD carrier	Isopropanol
Injection type:	Dry sample

Table 3.23 Automatic flash chromatography gradient method of Fr. D5-3 of *G. divaricata*

Gradient method		
Step	Time (min.)	%B
1	0	20
2	60	100

Upon monitoring by TLC under UV, five different fractions (Fr. D5-3-1~5) were obtained (Table 3.24), after concentration by evaporation under reduced pressure with a rotavapor.

Table 3.24 Column chromatography fractionation of Fr. D5-3 of *G. divaricata*

Fraction	Weight (mg)
Fr. D5-3-1	
Fr. D5-3-2	1530
Fr. D5-3-3	610
Fr. D5-3-4	380
Fr. D5-3-5	750

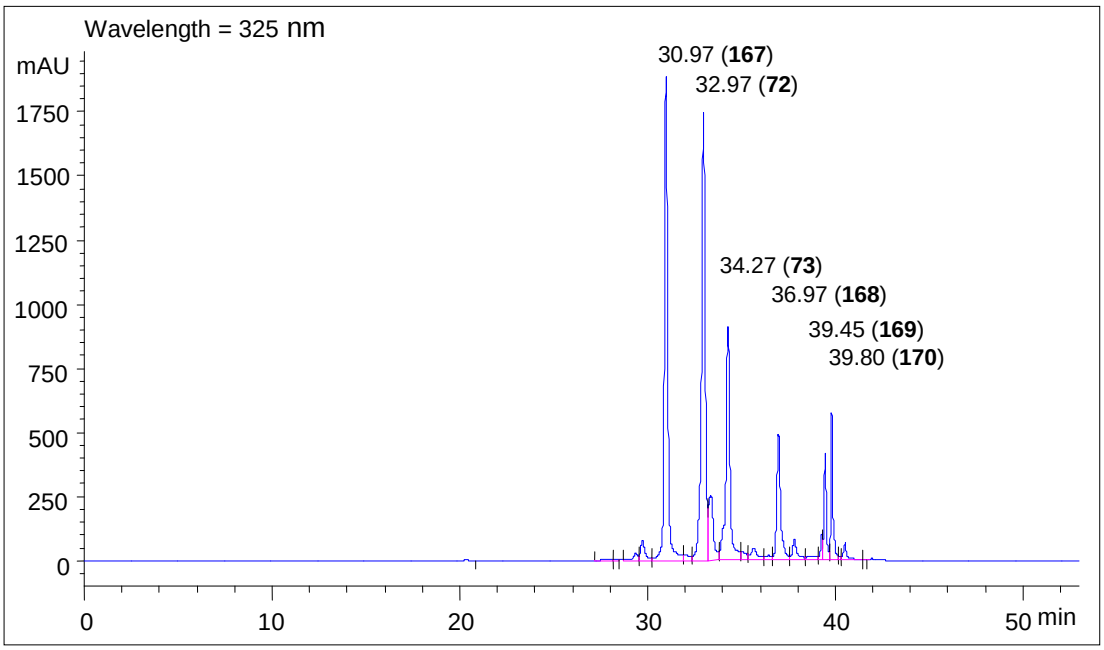
Fr. D5-3-2 (1530 mg) was dissolved in a small amount of methanol, and chromatographed over Sephadex LH-20 gel column eluting with a methanol/water gradient (50-100%) as solvent to afford three fractions (Fr. D5-3-2-1~3, Table 3.25).

Table 3.25 Column chromatography fractionation of Fr. D5-3-2 of *G. divaricata*

Fraction	Weight (mg)
Fr. D5-3-2-1	450
Fr. D5-3-2-2	680
Fr. D5-3-2-3	320

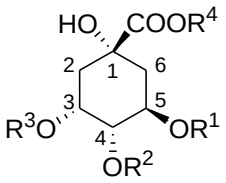
An analytical HPLC method was developed to analyse the constituents in the Fr. D5-3-2-2. In the HPLC spectrum (Figure 3.2), six major peaks were observed which were separated from each other well. The analytical HPLC conditions were the following: column, inertsil ODS-SP, 5 μm 4.6 × 250 mm, Shimadzu; solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, linear gradient mode 10-30% B in 0-30 min, 30-100% B in 30-45 min, 100-10% in 45-50 min; flow rate, 0.8 mL/min; detection, UV 325 nm; running time, 50 min.

Figure 3.2 HPLC chromatogram of the constituents in Fr. D5-3-2-2 of *G. divaricata*

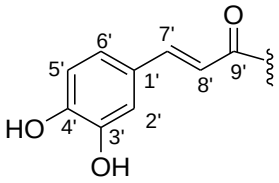


To isolate these six compounds, a preparative-HPLC method was developed. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, isocratic mode 20% B in 0-30 min, linear gradient mode 20-30% B in 30-55 min, 30-20% in 55-57 min; flow rate, 6 mL/min; detection, UV 325 nm; room temperature; injection volume, 250 μL per time; running time, 57 mins per injection; automatic fraction collector mode peak-based. About 100 mg of sample was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. Six eluates were collected and dried under high vacuum to afford compound **167** (44.3 mg), **72** (38.2 mg), **73** (26.3 mg), **168** (13.6 mg), **169** (9.4 mg) and **170** (10.5 mg), respectively.

Analysis of their spectral data, together with comparison with data available in the literature,[184,185,186,187] allowed to elucidate the structure of the compounds **167**, **72**-**73** and **168**-**170** as 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, methyl 3,4-dicaffeoylquinate, methyl 3,5-dicaffeoylquinate and methyl 4,5-dicaffeoylquinate, respectively.



quinate



caffeoyl

Compound	Name	R^1	R^2	R^3	R^4
167	3,4-dicaffeoylquinic acid	H	caffeoyl	caffeoyl	H
72	3,5-dicaffeoylquinic acid	caffeoyl	H	caffeoyl	H
73	4,5-dicaffeoylquinic acid	caffeoyl	caffeoyl	H	H
168	methyl 3,4-dicaffeoylquinate	H	caffeoyl	caffeoyl	Me
169	methyl 3,5-dicaffeoylquinate	caffeoyl	H	caffeoyl	Me
170	methyl 4,5-dicaffeoylquinate	H	caffeoyl	caffeoyl	Me

3,4-Dicaffeoylquinic acid 167: pale green powder; m.p. 168-169 °C; ESI-MS m/z: 515 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.57, 7.55 (2H, d, *J* = 16 Hz, H-7', 7"), 7.04, 7.02 (2H, d, *J* = 2 Hz, H-2', 2"), 6.93, 6.88 (2H, dd, *J* = 8, 2 Hz, H-6', 6"), 6.77, 6.73 (2H, d, *J* = 8 Hz, H-5', 5"), 6.29, 6.26 (2H, d, *J* = 16 Hz, H-8', 8"), 5.60-5.67 (1H, m, H-3), 5.00 (1H, dd, *J* = 3.3, 9 Hz, H-4), 4.31-4.41 (1H, m, H-5), 1.99-2.38 (4H, m, H-2 and H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 178.5 (COOH), 168.9 (C-9', 9"), 149.7 (C-4', 4"), 147.6 (C-7', 7"), 146.9 (C-3', 3"), 128.0 (C-1', 1"), 123.5 (C-6', 6"), 116.7 (C-5', 5"), 115.4 (C-2', 2"), 115.2 (C-8', 8"), 76.6 (C-4), 75.6 (C-1), 70.4 (C-3), 66.1 (C-5), 42.0 (C-2), 37.2 (C-6).

3,5-Dicaffeoylquinic acid 72: pale green powder; m.p. 169-170 °C; ESI-MS m/z: 515 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.62, 7.59 (2H, d, *J* = 16 Hz, H-7', 7"), 7.07 (2H, s, H-2', 2"), 6.97, 6.96 (2H, dd, *J* = 8.3, 2.2 Hz, H-6', 6"), 6.79, 6.77 (2H,d, *J* = 8.3 Hz, H-5', 5"), 6.36, 6.27 (2H, d, *J* = 16 Hz, H-8', 8"), 5.34-5.46 (2H, m, H-3 and H-5), 3.97 (1H, dd, *J* = 2.7, 7.5 Hz, H-4), 2.04-2.34 (4H, m, H-2 and H-6). ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 177.6 (COOH), 168.9, 168.4 (C-9', 9"), 149.6, 149.5 (C-4', 4"), 147.3, 147.0 (C-7', 7"), 146.8 (C-3', 3"), 127.9, 127.8 (C-1', 1"), 123.1, 123.0 (C-6', 6"), 116.4 (C-5', 5"), 115.6, 115.2 (C-2', 2"), 115.1 (C-8', 8"), 74.8 (C-1), 72.6 (C-3), 72.1 (C-5), 70.7 (C-4), 37.7 (C-6), 36.1 (C-2).

4,5-Dicaffeoylquinic acid 73: pale green powder; m.p. 170-171 °C; ESI-MS m/z: 515 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.56, 7.47 (2H, d, *J* = 16 Hz, H-7', 7"), 6.96 (2H, s, H-2', 2"), 6.86, 6.84 (2H, d, *J* = 7.2 Hz, H-6', 6"), 6.72, 6.69 (2H, d, *J* = 7.2 Hz, H-5', 5"), 6.25, 6.15 (2H, d, *J* = 16 Hz, H-8', 8"), 5.50-5.68 (1H, m, H-5), 5.00-5.20 (1H, m, H-4), 4.27-4.42 (1H, m, H-3), 1.93-2.38 (4H, m, H-2 and H-6). ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 177.1 (COOH), 168.5, 168.3 (C-9', 9"), 149.6 (C-4', 4"), 147.7, 147.5 (C-7', 7"), 146.6 (C-3', 3"), 127.6 (C-1', 1"), 123.1 (C-6', 6"), 116.4 (C-5', 5"), 115.2 (C-2', 2"), 114.7 (C-8', 8"), 76.2 (C-4), 75.8 (C-1), 69.4 (C-3), 69.0 (C-5), 39.4 (C-6), 38.3 (C-2).

Methyl 3,4-dicaffeoylquinate 168: pale yellow powder; m.p. 142-143 °C; ESI-MS m/z: 529 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.55, 7.50 (2H, d, *J* = 16 Hz, H-7', 7"), 7.01 (2H, s, H-2', 2"), 6.87 (2H, d, *J* = 7.7 Hz, H-6', 6"), 6.74 (2H, d, *J* = 7.7 Hz, H-5', 5"), 6.27, 6.22 (2H, d, *J* = 16 Hz, H-8', 8"), 5.52-5.66 (1H, m, H-3), 4.95-5.15 (1H, m, H-4), 4.20-4.35 (1H, m, H-5), 3.73 (3H, s, $COOC\underline{H}_3$), 1.96-2.35 (4H, m, H-2 and H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 176.1 ($\underline{C}OOCH_3$), 168.5 (C-9', 9"), 149.6 (C-4', 4"), 147.3 (C-7', 7"), 146.8 (C-3', 3"), 127.7 (C-1', 1"), 123.2, 123.1 (C-6', 6"), 116.4 (C-5', 5"), 115.1 (C-2', 2"), 115.0, 114.8 (C-8', 8"), 75.6 (C-4), 75.2 (C-1), 69.8 (C-3), 69.8 (C-5), 41.3 (C-2), 36.8 (C-6).

Methyl 3,5-dicaffeoylquinate 169: pale yellow powder; m.p. 143-144 °C; ESI-MS m/z: 529 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.62, 7.55 (2H, d, *J* = 16 Hz, H-7', 7"), 7.07 (2H, d, *J* = 2 Hz, H-2', 2"), 6.99, 6.96 (2H, d, *J* = 8 Hz, H-6', 6"), 6.80, 6.77 (2H, d, *J* = 8 Hz, H-5', 5"), 6.35, 6.22 (2H, d, *J* = 16 Hz, H-8', 8"), 5.36-5.43 (1H, m, H-5), 5.27-5.35 (1H, m, H-3), 3.98 (1H, dd, *J* = 7.5, 2.7 Hz, H-4), 3.69 (3H, s, $COOC\underline{H}_3$), 2.11-2.36 (4H, m, H-2 and H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 175.6 ($\underline{C}OOCH_3$), 168.7, 167.9 (C-9', 9"), 149.8, 149.6 (C-4', 4"), 147.4, 147.1 (C-7', 7"), 146.9, 146.8 (C-3', 3"), 127.9, 127.6 (C-1', 1"), 123.1, 123.0 (C-6', 6"), 116.6, 116.5 (C-5', 5"), 115.5, 115.1 (C-2', 2"), 115.1, 114.8 (C-8', 8"), 74.6 (C-1), 72.2 (C-3), 72.0 (C-5), 69.7 (C-4), 53.0 ($COO\underline{C}H_3$), 37.0 (C-6), 35.6 (C-2).

Methyl 4,5-dicaffeoylquinate 170: pale yellow powder; m.p. 145-146 °C; ESI-MS m/z: 529 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.61, 7.51 (2H, d, *J* = 16 Hz, H-7', 7"), 7.03 (2H, s, H-2', 2"), 6.91-6.93 (2H, m, H-6', 6"), 6.78, 6.75 (2H, d, *J* = 8 Hz, H-5', 5"), 6.30, 6.18 (2H, d, *J* = 16 Hz, H-8', 8"), 5.48-5.59 (1H, m, H-5), 5.08-5.15 (1H, m, H-4), 4.30-4.39 (1H, m, H-3), 3.72 (3H, s, $COOC\underline{H}_3$), 1.99-2.36 (m, H-2 and H-6). ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 175.2 ($\underline{C}OOCH_3$), 168.5, 167.9 (C-9', 9"), 149.8, 149.7 (C-4', 4"), 147.7 (C-7', 7"), 146.8 (C-3', 3"), 127.7, 127.5 (C-1', 1"), 123.2 (C-6', 6"), 116.5 (C-5', 5"), 115.1 (C-2', 2"), 114.7, 114.5 (C-8', 8"), 75.8 (C-4), 74.8 (C-1), 69.1 (C-3), 68.6 (C-5), 53.1 ($COO\underline{C}H_3$), 38.5 (C-6), 38.4 (C-2).

3.2.3.2.11 Isolation of 5-O-*p*-coumaroylquinic acid **171**, methyl 5-O-caffeoylquinate **172** and 5-O-feruloylquinic acid **173**

Fr. D5-3-2-1 (450 mg) was subjected to column chromatography over silica gel eluted by a solvent gradient system consisting of $CHCl_3$-MeOH (Table 3.26) to yield three fractions (Fr. D5-3-2-1-1~3, Table 3.27).

Table 3.26 Programme for fractionation of Fr. D5-3-2-1 of *G. divaricata* by column chromatography

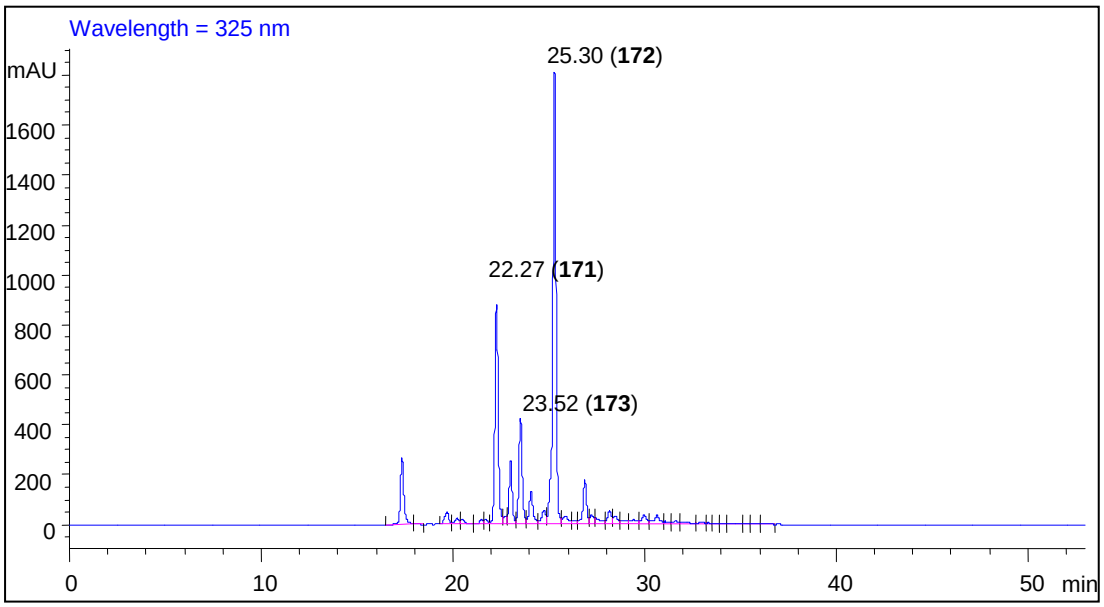
Mobile phase	Gradient elution	Solvent volume (mL)
$CHCl_3$/MeOH	10:1	1100
$CHCl_3$/MeOH	8:1	1350
$CHCl_3$/MeOH	8:2	800

Table 3.27 Column chromatography fractionation of Fr. D5-3-2-1 of *G. divaricata*

Fraction	Weight (mg)
Fr. D5-3-2-1-1	66
Fr. D5-3-2-1-2	102
Fr. D5-3-2-1-3	186

Fr. D5-3-2-1-2 (102 mg) was analyzed by HPLC before its further purification. Five mg of Fr. D5-3-2-1-2 was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. The analytical HPLC conditions were the following: column, inertsil ODS-SP, 5 μm 4.6 × 250 mm, Shimadzu; solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, linear gradient mode 10-30% B in 0-30 min, 30-100% B in 30-45 min, 100-10% in 45-50 min; flow rate, 0.8 mL/min; detection, UV 325 nm; running time, 50 min. In the HPLC spectrum (Figure 3.3), two major peaks (Rt 22.27 and Rt 25.30) were observed and these two compounds were targeted in the subsequent separation.

Figure 3.3 HPLC chromatogram of the constituents in Fr. D5-3-2-1-2 of *G. divaricata*

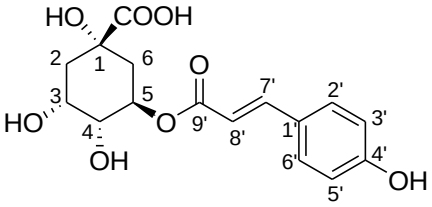


To isolate the target compounds, a preparative-HPLC method was developed. The prep-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, linear gradient mode 15-20% B in 0-25 min, 20-50% B in 25-28 min, 50-15% in 28-30 min; flow rate, 6 mL/min; detection, UV 325 nm; room temperature; injection volume, 120 μL per time; running time, 30 mins per injection; automatic fraction collector mode peak-based. About 50 mg of sample was dissolved in 1 mL MeOH and filtered with 0.45 μm filter prior to injection. Four eluates named Fr. D5-3-2-1-2-1~4 were collected (Table 3.28). Fr. D5-3-2-1-2-1 and Fr. D5-3-2-1-2-4 were dried under high vacuum to afford compounds **171** (1.2 mg) and **172** (8.7 mg), respectively.

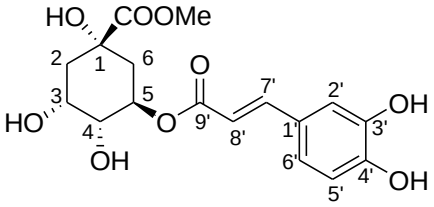
Table 3.28 Column chromatography fractionation of Fr. D5-3-2-1-2 of *G. divaricata*

Fraction	Weight (mg)
Fr. D5-3-2-1-2-1	1.2
Fr. D5-3-2-1-2-2	0.3
Fr. D5-3-2-1-2-3	0.6
Fr. D5-3-2-1-2-4	8.7

Analysis of their spectral data, together with comparison with data available in the literatures,[188,189] allowed to elucidate the structure of compounds **171-172** as 5-O-*p*-coumaroylquinic acid and methyl 5-O-caffeoylquinate, respectively.



171



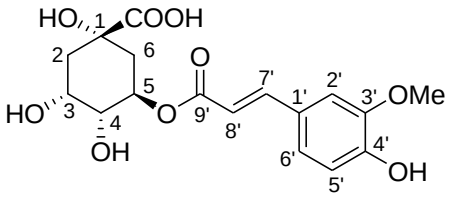
172

5-O-*p*-Coumaroylquinic acid 171: white powder; m.p. 207-208 °C; ESI-MS m/z: 337 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.63 (1H, d, *J* = 16.0 Hz, H-7'), 7.47 (2H, d, *J* = 8.8 Hz, H-2' and H-6'), 6.81 (2H, d, *J* = 8.8 Hz, H-3' and C-5'), 6.33 (1H, d, *J* = 16.0 Hz, H-8'), 5.28-5.38 (m, H-5), 4.12-4.20 (1H, m, H-3), 3.73 (1H, dd, *J* = 8.3, 2.8 Hz, H-4), 2.01-2.25 (4H, m, H-2 and H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 177.1 (COOH), 168.5 (C-9'), 160.8 (C-4'), 146.5 (C-7'), 130.9 (C-2' and C-6'), 127.0 (C-1'), 116.5 (C-3' and C-5'), 115.0 (C-8'), 76.0 (C-1), 73.1 (C-5), 71.7 (C-3), 71.0 (C-4), 38.5 (C-6), 37.9 (C-2).

Methyl 5-O-caffeoylquinate 172: white powder; m.p. 162-163 °C; ESI-MS m/z: 367 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.52 (1H, d, *J* = 16.0 Hz, H-7'), 7.04 (1H, d, *J* = 2.2 Hz, H-2'), 6.95 (1H, dd, *J* = 8.0, 2.2 Hz, H-6'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), 6.22 (1H, d, *J* = 16.0 Hz, H-8'), 5.24-5.30 (1H, m, H-5), 4.12-4.15 (1H, m, H-3), 3.72-3.75 (1H, m, H-4), 3.69 (3H, s, $COOCH_3$), 1.97-2.25 (4H, m, H-2 and H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 175.4 ($COOCH_3$), 168.3 (C-9'), 149.7 (C-4'), 147.2 (C-3'), 146.9 (C-7'), 127.6 (C-1'), 123.0 (C-6'), 116.5 (C-5'), 115.1 (C-2'), 115.0 (C-8'), 75.8 (C-1), 72.5 (C-5), 72.1 (C-4), 70.3 (C-3), 53.0 ($COOCH_3$), 38.0 (C-6), 37.7 (C-2).

Fr. D5-3-2-1-2-2 and Fr. D5-3-2-1-2-3 were combined for further purification, by a preparative-HPLC method. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, isocratic mode 21% B; flow rate, 6 mL/min; detection, UV 325 nm; room temperature; injection volume, 60 μL per time; running time, 30 mins per injection; automatic fraction collector mode peak-based. The eluate was collected and dried under high vacuum to afford compound **173** (0.5 mg).

Analysis of its spectral data, together with comparison of data available in the literature,[190] allowed to elucidate the structure of compound **173** as 5-O-feruloylquinic acid.



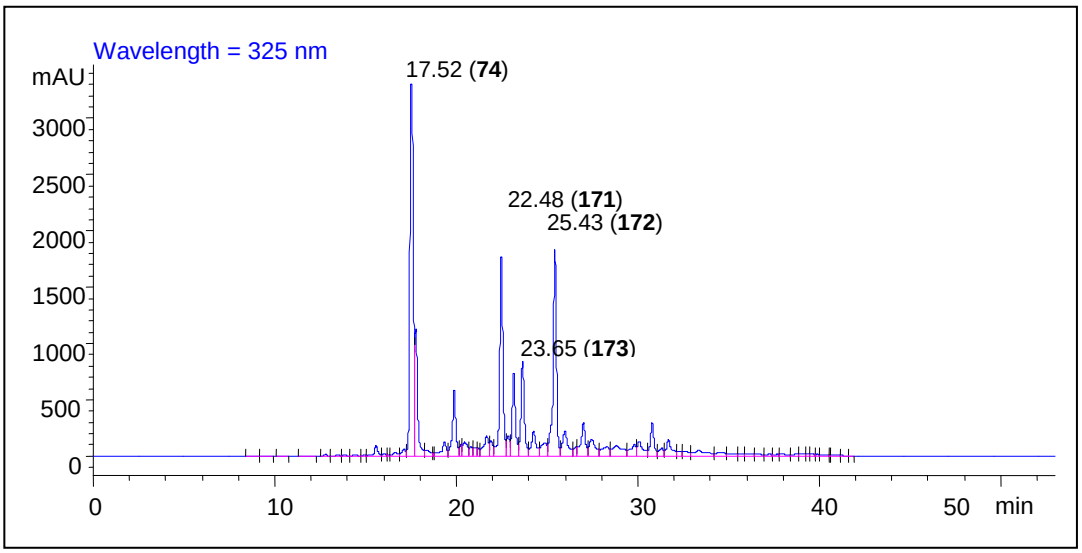
173

5-O-Feruloylquinic acid 173: white powder; m.p. 196-197 °C; ESI-MS m/z: 367 $[M-H]^-$; ^1H NMR δ ppm (MeOH-d_4, 300 MHz): 7.62 (1H, d, *J* = 16.0 Hz, H-7'), 7.19 (1H, s, H-2'), 7.08 (1H, d, *J* = 7.4 Hz, H-6'), 6.81 (1H, d, *J* = 7.4 Hz,H-5'), 6.36 (1H, d, *J* = 16.0 Hz, H-8'), 5.30-5.40 (1H, m, H-5), 4.16-4.22 (1H, m, H-3), 3.89 (3H, s, OCH_3), 3.69-3.77 (1H, m, H-4), 1.87-2.21 (4H, m, H-2 and H-6).

3.2.3.2.12 Isolation of 5-O-caffeoylquinic acid **74**

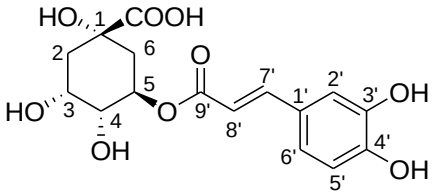
Fr. D5-3-2-1-3 (186 mg) was analyzed by HPLC before further purification. Five mg of Fr. D5-3-2-1-3 was dissolved in 1 mL MeOH and filtered with a 0.45 μm filter prior to injection. The analytical HPLC conditions were the following: column, inertsil ODS-SP, 5 μm 4.6 × 250 mm, Shimadzu; solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, linear gradient mode 10-30% B in 0-30 min, 30-100% B in 30-45 min, 100-10% in 45-50 min; flow rate, 0.8 mL/min; detection, UV 325 nm; running time, 50 min. In the HPLC spectrum (Figure 3.4), three major peaks (Rt 17.52, 22.48 and 25.43 min) were observed with good resolution. The peak with retention time 17.52 min was targeted in a subsequent separation.

Figure 3.4 HPLC chromatogram of the constituents in Fr. D5-3-2-1-3 of *G. divaricata*



A preparative-HPLC method was developed to isolate the target compound. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, linear gradient mode 16-20% B in 0-25 min, 20-50% B in 25-27 min, 50-16% in 27-28 min; flow rate, 6 mL/min; detection, UV 325 nm; room temperature; injection volume, 150 μL per time; running time, 28 mins per injection; automatic fraction collector mode peak-based. About 50 mg of sample was dissolved in 1 mL MeOH and filtered with a 0.45 μm filter prior to injection. The eluate was collected and dried under high vacuum to afford compound **74** (5.0 mg).

Analysis of the spectral data, together with comparison with data available in the literature,[189] allowed to elucidate the structure of the compound **74** as 5-O-caffeoylquinic acid.



74

5-O-Caffeoylquinic acid 74: white powder; m.p. 207-208 °C; ESI-MS m/z: 353 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.56 (1H, d, *J* = 16.0 Hz, H-7'), 7.04 (1H, d, *J* = 1.7 Hz, H-2'), 6.95 (1H, d, *J* = 8.3, H-6'), 6.77 (1H, d, *J* = 8.3 Hz, H-5'), 6.27 (1H, d, *J* = 16.0 Hz, H-8'), 5.29-5.36 (1H, m, H-5), 4.12-4.20 (1H, m, H-3), 3.70-3.75 (1H, m, H-4), 2.01-2.08 (2H, m, H-2), 2.16-2.21 (2H, m, H-6); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 177.0 (COOH), 168.7 (C-9'), 149.6 (C-4'), 147.1 (C-3'), 146.8 (C-7'), 127.8 (C-1'), 123.0 (C-6'), 116.5 (C-5'), 115.3 (C-2'), 115.2 (C-8'), 76.1 (C-1), 73.5 (C-5), 72.0 (C-4), 71.3 (C-3), 38.8 (C-6), 38.2 (C-2).

3.2.3.2.13 Isolation of ethyl 4,5-dicaffeoylquinate **174**

Fr. D5-3-2-3 (320 mg) was subjected to column chromatography over silica gel eluted by a solvent gradient system consisting of $CHCl_3$-MeOH (Table 3.29) to yield four fractions (Fr. D5-3-2-3-1~4, Table 3.30).

Table 3.29 Programme for fractionation of Fr. D5-3-2-3 of *G. divaricata* by column chromatography

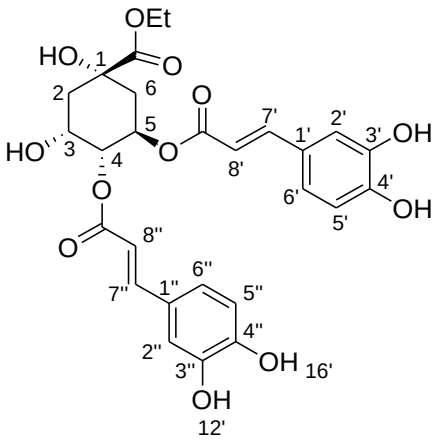
Mobile phase	Gradient elution	Solvent volume (mL)
$CHCl_3$/MeOH	20:1	1050
$CHCl_3$/MeOH	10:1	1100
$CHCl_3$/MeOH	9:1	1000
$CHCl_3$/MeOH	6:1	700

Table 3.30 Column chromatography fractionation of Fr. D5-3-2-3 of *G. divaricata*

Fraction	Weight (mg)
Fr. D5-3-2-3-1	25
Fr. D5-3-2-3-2	26
Fr. D5-3-2-3-3	33
Fr. D5-3-2-3-4	46

Fr. D5-3-2-3-3 (33 mg) was additionally purified by prep-HPLC. Fr. D5-3-2-3-3 was dissolved in 1 mL MeOH and filtered with a 0.45 μm filter prior to injection. The preparative-HPLC conditions were: C18 column with guard cartridge, 21.2 mm ID × 150 mm (5 μm); solvents for the mobile phase, water-0.1% formic acid (A) and MeCN (B); elution, isocratic 35% B in 15 min; flow rate, 6 mL/min; detection, UV 325 nm; room temperature; injection volume, 80 μL per time; running time, 15 mins per injection; automatic fraction collector mode peak-based. The eluate from 12.4 to 12.7 min was collected and dried under high vacuum to afford compound **174** (1.0 mg).

Analysis of the spectral data, together with comparison with data available in the literature,[191] allowed to elucidate the structure of compound **174** as ethyl 4,5-dicaffeoylquinate.



174

Ethyl 4,5-dicaffeoylquinate 174: white powder; m.p. 118-119 °C; ESI-MS m/z: 543 $[M-H]^-$; ^{1}H NMR δ ppm (MeOH-d_4, 300 MHz): 7.61, 7.51 (2H, d, *J* = 16.0 Hz, H-7′, 7″), 7.04, 7.01 (2H, d, *J* = 1.7 Hz, H-2′, 2″), 6.90-6.95 (2H, m, H-6′, 6″), 6.76 (2H, d, *J* = 8.3 Hz, H-5′, 5″), 6.30, 6.18 (2H, d, *J* = 16.0 Hz, H-8′, 8″), 5.50-5.57 (1H, m, H-5), 5.09-5.13 (1H, m, H-4), 4.34-4.36 (1H, m, H-3), 4.13-4.21 (2H, m, $COOC\underline{H}_2CH_3$), 2.03-2.36 (4H, m, H-2 and H-6), 1.26 (3H, t, *J* = 7.2 Hz, $COOCH_2C\underline{H}_3$); ^{13}C NMR δ ppm (MeOH-d_4, 75 MHz): 174.7 ($\underline{C}OOEt$), 168.5, 167.9 (C-9′, 9″), 149.8, 149.7 (C-4′, 4″), 147.7, 147.6 (C-7′, 7″), 146.8 (C-3′, 3″), 127.6, 127.5 (C-1′, 1″), 123.1 (C-6′, 6″), 116.5 (C-2′, 2″), 115.1 (C-5′, 5″), 114.6, 114.5 (C-8′, 8″), 75.6 (C-1), 74.8 (C-5), 69.1 (C-4), 68.5 (C-3), 62.7 ($COO\underline{C}H_2CH_3$), 38.4 (C-6), 38.3 (C-2), 14.3 ($COOCH_2\underline{C}H_3$).

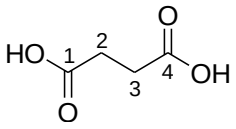
3.3 Results and Discussion

Gynura divaricata is a traditional Chinese medicine which is called "Bai Bei San Qi" in Chinese. The chemical constituents of *G. divaricata* include alkaloids,[22] cerebrosides,[41,42,43] phenolics,[31] polysaccharides and oligosaccharides.[192]

In the present study, one organic acid (**94**) and three of its derivatives (**161-163**), four phenolic acids (**79**, **160, 164-165**), one coumarin (**166**), five flavonoids (**35**-**36**, **38**, **40**-**41**), and eleven quinic acid derivatives (**72**-**74**, **167-174**) were isolated from the aerial parts of *G. divaricata*. All the compounds were characterized by spectrometric methods (NMR, MS). It should be noted that regarding these constituents, compounds **36**, **73**-**74**, **79**, **94** and **160-174** were isolated for the first time from this plant.

3.3.1 Organic acid and its derivatives from aerial parts of *G. divaricata*

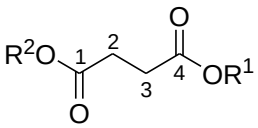
3.3.1.1 Organic acid compound: succinic acid **94**



94

Compound **94** was obtained as needle crystals. The ESI mass spectrum of compound **94** displayed a pseudomolecular ion peak at m/z 117 which may correspond to the fragment $[M-H]^-$. The molecular formula of $C_4H_6O_4$ was deduced. The ^1H NMR spectrum of **94** disclosed the presence of a single resonance signal at δ 2.57. On the other hand, the ^{13}C NMR spectrum of **94** exhibited two signals at δ 174.1, which was deduced to result from a carboxylic acid, and 29.3, which could be attributed to a carbon connected with the carboxylic acid. According to its molecular formula, compound **94** was elucidated as succinic acid. The assigned structure was in full agreement with data found in the Spectral Database for Organic Compounds (SDBS).[175]

3.3.1.2 Organic acid derivatives: ethyl methyl succinate **161,** methyl succinate **162** and ethyl succinate **163**



161: R^1 = Me, R^2 = Et
162: R^1 = H, R^2 = Me
163: R^1 = H, R^2 = Et

Compound **161** was obtained as a colorless liquid. The ^1H NMR spectrum of **161** contained one methyl proton signal at δ 1.27 (3H, t), one singlet of a methyl substituent at δ 3.71 (3H, s) which was coupled to an oxygen. Besides, a methylene was observed at δ 4.16 as quartet suggesting the presence of the $–OCH_2CH_3$ moiety. Additionally, one

multiplet (4H) at δ 2.60-2.73 could be assigned to two nonequivalent methylenes adjacent to the carbonyl. In the ^{13}C NMR spectrum of **161**, two carbonyl signals at δ 172.8 (C-4) and 172.3 (C-1), and a carbon signal (2C) at δ 28.9 were visible. These spectral data resulted in the assignment of the structure of compound **161** as ethyl methyl succinate.

Compound **162** was obtained as a white powder. The ESI mass spectrum of compound **162** displayed an ion peak at m/z 150 which could correspond to the fragment $[M+NH_4]^+$, suggesting a molecular formula of $C_5H_8O_4$. The ^{1}H NMR spectrum of **162** contained a methoxy signal at δ 3.57 (3H, s), one multiplet (4H) at δ 2.49-2.51. In the ^{13}C NMR spectrum of **162**, four carbon signals were assigned to two carbonyls at δ 174.8 and 173.5, one methoxy at δ 50.9 and two methylenes at δ 28.5. These spectral data led to the structure assignment of compound **162** as methyl succinate.

Compound **163** was obtained as a white powder. The ESI mass spectrum of compound **163** showed an ion peak at m/z 145 corresponding to the fragment $[M-H]^-$, suggesting a molecular formula of $C_6H_{10}O_4$. The ^{1}H NMR spectrum of **163** was similar as that of ethyl methyl succinate **161** except the absence of a methoxy signal around δ 3.71. Compared with the ^{13}C NMR spectrum of **161**, the oxygenated methyl at δ 52.1 was missing. Based on these observations, compound **163** could be elucidated as ethyl succinate.

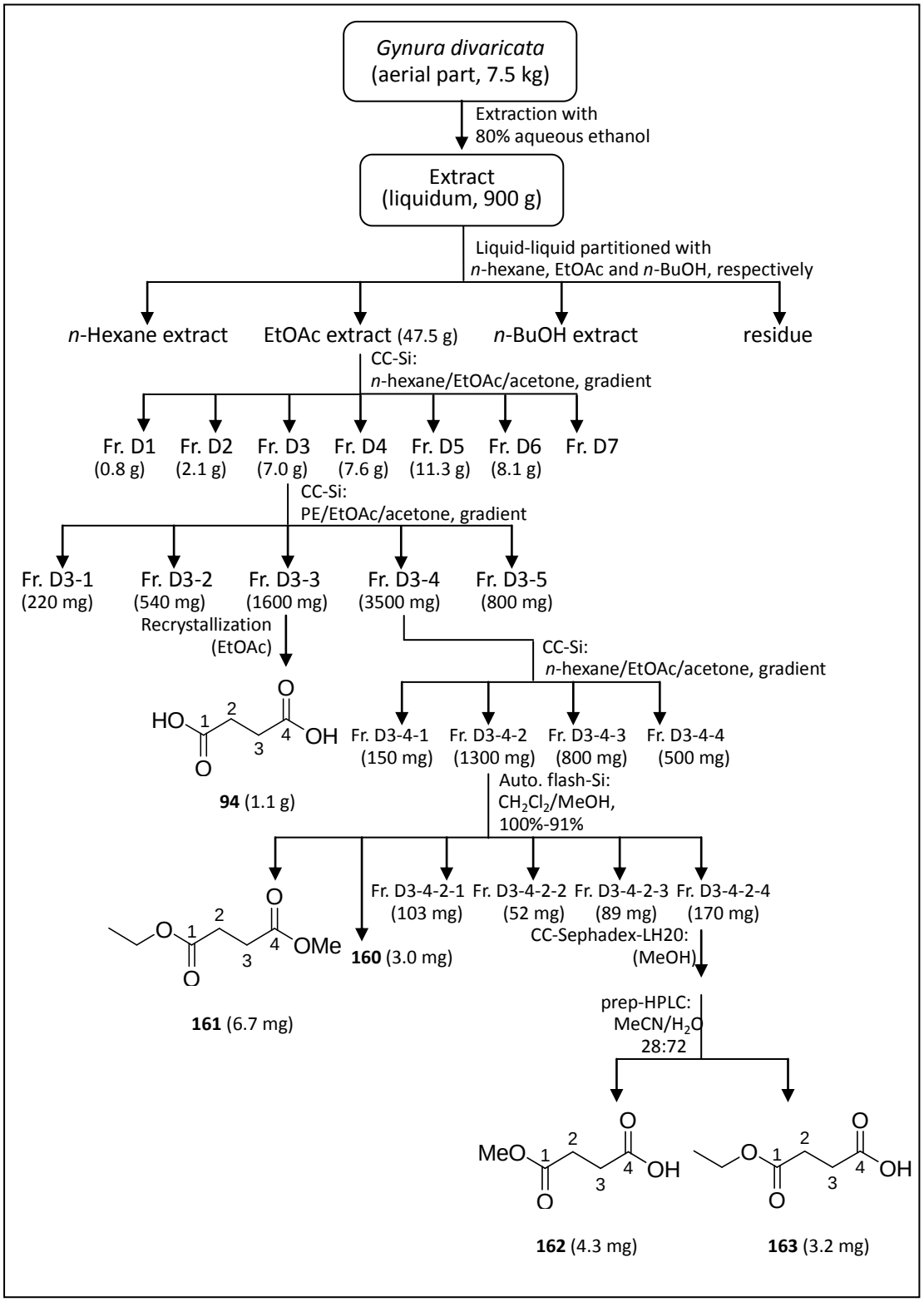
The comparison of characteristic NMR data of compounds **94** and **161-163** with literature data is listed in Table 3.31.[175]

Table 3.31 Comparison of characteristic NMR data of succinic acid **94** (300/75 MHz, D_2O), ethyl methyl succinate **161** (300/75 MHz, $CDCl_3$), methyl succinate **162** (300/75 MHz, $CDCl_3$) and ethyl succinate **163** (300/75 MHz, MeOH-d_4) with literature data (400/100 MHz, DMSO-d_6)[175]

Position	Compounds isolated in this work				Literature data
	94	**161**	**162**	**163**	succinic acid
^{1}H NMR					
COOC<u>H</u>$_2$CH$_3$		4.16 (2H, q)		4.16 (2H, q)	
COOCH$_2$C<u>H</u>$_3$		1.27 (3H, t)		1.25 (3H, t)	
COOC<u>H</u>$_3$		3.71 (3H, s)	3.57 (3H, s)		
H-2	2.57	2.60-2.73	2.49-2.51	2.38-2.69	2.43 (s)
H-3	(4H, s)	(4H, m)	(4H, m)	(4H, m)	
^{13}C NMR					
C-1	174.1	172.3	173.5	176.4	173.5
C-2	29.3	28.9	28.5	28.5	28.8
C-3	29.3	28.9	28.5	28.5	28.8
C-4	174.1	172.8	174.8	176.4	173.5
COO<u>C</u>H$_2$CH$_3$		61.0		60.9	
COOCH$_2$<u>C</u>H$_3$		14.2		14.1	
COO<u>C</u>H$_3$		52.1	50.9		

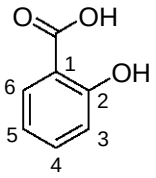
Scheme 3.1 describes the complete isolation of compounds **94** and **161**-**163** from the aerial parts of *G. divaricata*.

Scheme 3.1 The complete isolation of compounds **94** and **161**-**163** from *G. divaricata*



3.3.2 Phenolic acids from aerial parts of *G. divaricata*

3.3.2.1 Phenolic acid compound: salicylic acid **160**



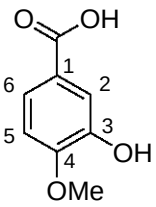
160

Compound **160** was obtained as a white powder. The ESI mass spectrum of compound **160** showed an ion peak at m/z 137 corresponding to the fragment $[M-H]^-$, suggesting a molecular formula of $C_7H_6O_3$. The ^1H NMR spectrum of compound **160** displayed four aromatic protons at δ 7.85 (1H, dd, *J* = 8.2, 1.7 Hz, H-6), 7.46 (1H, ddd, *J* = 8.3, 7.2, 1.7 Hz, H-4), 6.91 (1H, ddd, *J* = 8.2, 7.2, 1.1 Hz, H-5) and 6.87 (1H, dd, *J* = 8.3, 1.1 Hz, H-3). In its ^{13}C NMR spectrum, compound **160** exhibited a signal at δ 173.5 for a carbonyl carbon and 163.2 for an oxygenated quaternary carbon (C-2). Additionally, five aromatic carbon signals were seen at δ 136.6 (C-4), 131.5 (C-6), 120.1 (C-5), 118.1 (C-3) and 113.9 (C-1), respectively. The above spectral data revealed the structure of compound **160** as salicylic acid. The spectral data of the compound isolated in the present study match well with those reported earlier.[176] The comparison of characteristic NMR data of compound **160** with literature data is listed in Table 3.32.[176]

Table 3.32 Comparison of characteristic ^1H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of salicylic acid **160** with literature data (500/125 MHz, MeOH-d_4)[176]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1		113.9		113.2
2		163.2		163.1
3	6.87 (1H, dd, *J* = 8.3, 1.1 Hz)	118.1	6.95 (1H, d, *J* = 8.2 Hz)	118.1
4	7.46 (1H, ddd, *J* = 8.3, 7.2, 1.7 Hz)	136.6	7.54 (1H, dt, *J* = 8.2, 1.6 Hz, H-4)	136.8
5	6.91 (1H, ddd, *J* = 8.2, 7.2, 1.1 Hz)	120.1	6.96 (1H, d, *J* = 8.2 Hz, H-5)	119.9
6	7.85 (1H, dd, *J* = 8.2, 1.7 Hz)	131.5	7.90 (1H, dd, *J* = 8.2, 1.6 Hz, H-6)	131.2
COOH		173.5		172.7

3.3.2.2 Phenolic acid compound: isovanillic acid **164**



164

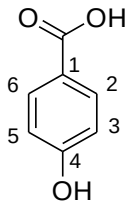
Compound **164** was obtained as a white powder. The ESI mass spectrum of compound **164** showed an ion peak at m/z 167 corresponding to the fragment $[M-H]^-$, suggesting a molecular formula of $C_8H_8O_4$. The ^1H NMR spectrum of compound **165** displayed three aromatic proton signals at 7.47 (1H, dd, *J* = 8.3, 1.7 Hz), 7.47 (1H, d, *J* = 1.7 Hz) and 6.75 (1H, d, *J* = 8.7 Hz) assignable to an ABX coupling system. Furthermore, its ^{13}C NMR spectral data were corresponding with those of isovanillic acid reported.[178]

The comparison of characteristic NMR data of compound **164** with literature data[178] is listed in Table 3.33.

Table 3.33 Comparison of characteristic ^1H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of isovanillic acid **164** with literature data (500/125 MHz, MeOH-d_4)[178]

Position	This work		Literature data	
	^1H	^{13}C	^1H	^{13}C
1		121.8		122.2
2	7.47 (1H, d, *J* = 1.7 Hz)	114.5	7.56 (1H, d, *J* = 1.9 Hz)	114.5
3		147.3		146.8
4		151.3		150.7
5	6.75 (1H, d, *J* = 8.7 Hz)	112.4	6.90 (1H, d, *J* = 8.0 Hz)	112.6
6	7.48 (1H, dd, *J* = 8.7, 1.7 Hz)	123.9	7.63 (1H, dd, *J* = 8.0, 1.9 Hz)	124.7
COOH		168.8		168.9
OCH_3	3.81 (3H, s)	55.0	3.94 (3H, s, H-8)	56.1

3.3.2.3 Phenolic acid compound: 4-hydroxybenzoic acid **79**



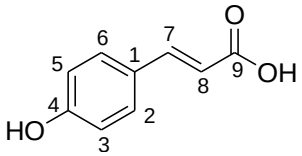
79

Compound **79** was isolated as a white powder. The ESI Mass spectrum of compound **79** displayed an ion peak at m/z 137 $[M-H]^-$, suggesting a molecular formula of $C_7H_6O_3$. In the ^1H NMR spectrum of compound **79**, two doublets in the aromatic region at δ 7.77 (2H, *J* = 8.3 Hz, H-2, 6) and δ 6.71 (2H, *J* = 8.3 Hz, H-3, 5) were assigned to the presence of a 1,4-disubstituted aromatic ring. By comparison of characteristic NMR data of compound **79** with data of compound **79** previously discussed in Chapter 2 (Table 3.34), the structure of compound **79** present isolated was elucidated as 4-hydroxybenzoic acid.

Table 3.34 Comparison of characteristic NMR (300/75 MHz, MeOH-d_4) data of 4-hydroxybenzoic acid **79** with identified one from *G. bicolor* (300/75 MHz, DMSO-d_6).

Position	Compound **79** from *Gynura divaricata*		4-Hydroxybenzoic acid from *Gynura bicolor*	
	^{1}H	^{13}C	^{1}H	^{13}C
1		121.7		122.0
2	7.77 (1H, d, *J* = 8.3 Hz)	131.7	7.77 (1H, dd, *J* = 8.8, 3.9 Hz)	132.1
3	6.71 (1H, d, *J* = 8.3 Hz)	114.7	6.80 (2H, dd, *J* = 8.8, 3.9 Hz)	115.7
4		161.9		162.1
5	6.71 (1H, d, *J* = 8.3 Hz)	114.7	6.80 (2H, dd, *J* = 8.8, 3.9 Hz)	115.7
6	7.77 (1H, d, *J* = 8.3 Hz)	131.7	7.77 (1H, dd, *J* = 8.8, 3.9 Hz)	132.1
COOH		169.0		167.7

3.3.2.4 Phenolic acid compound: *p*-coumaric acid **165**



165

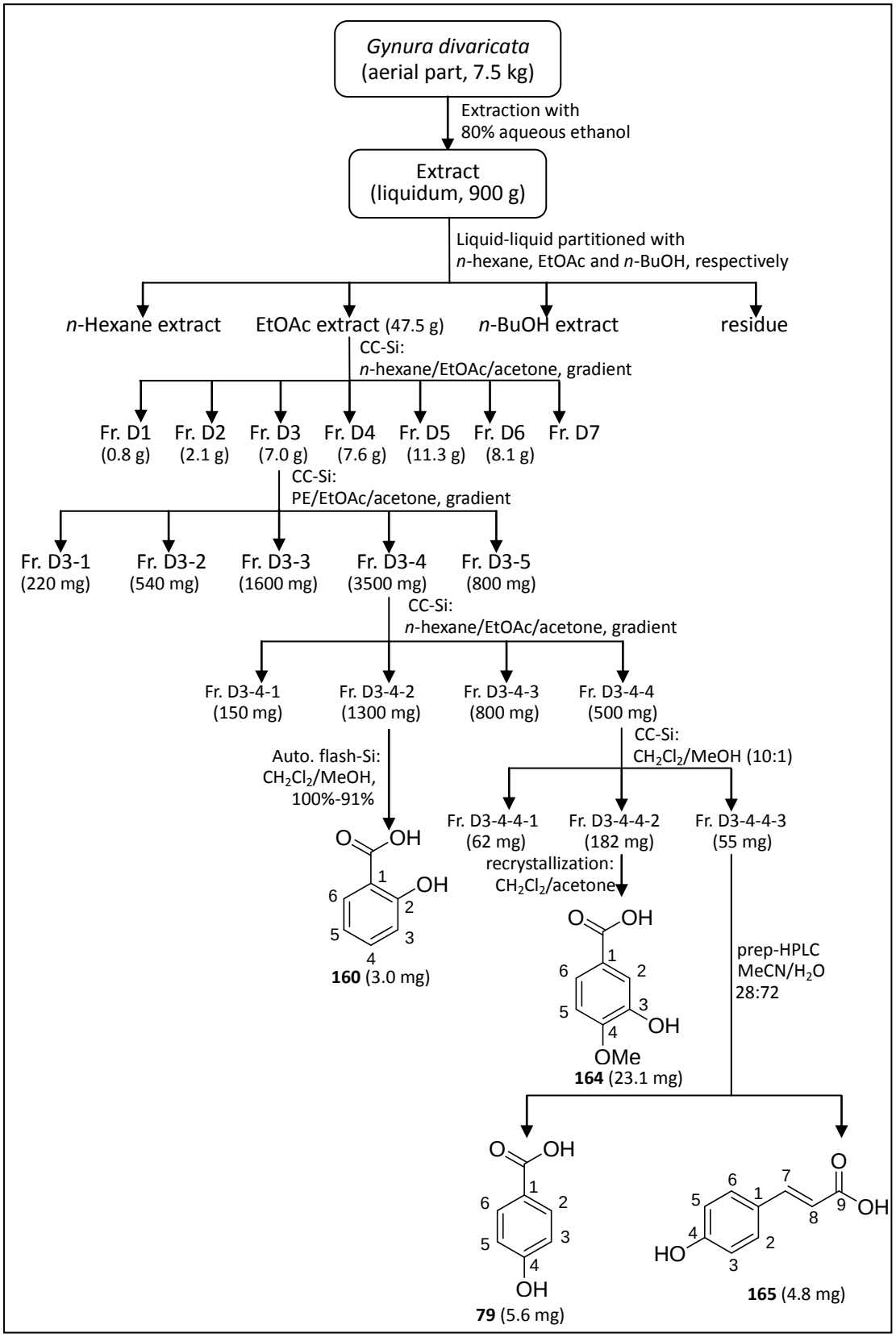
Compound **165** was obtained as a white powder. The ESI mass spectrum of compound **165** displayed an ion peak at m/z 163 $[M-H]^-$, suggesting its molecular formula as $C_9H_8O_3$. The ^{1}H NMR spectrum of compound **165** showed two doublets at δ 7.52 (1H, *J* = 15.9 Hz) and δ 6.20 (1H, *J* = 15.9 Hz) revealing the presence of a pair of olefinic protons and their coupling constants suggested a trans configuration. Two doublets centered at δ 7.37 (*J* = 8.8 Hz) and 6.73 (*J* = 8.8 Hz), each integrating for two protons, indicated a 1,4-disubstituted aromatic ring. On the other hand, the ^{13}C NMR of **165** displayed seven carbon signals, of which, the signals at δ 169.7 and 159.9 were assignable to a carbonyl and oxygenated aromatic carbon, respectively. The above spectral features were in close agreement to those observed for trans-*p*-coumaric acid. The comparison of characteristic NMR data of compound **165** with literature data is listed in Table 3.35.[177]

Table 3.35 Comparison of characteristic ^{1}H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of *p*-coumaric acid **165** with literature data (400/100 MHz, DMSO-d_6)[177]

Position	This work		literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		125.9		124.4
2,6	7.37 (2H, d, *J* = 8.8 Hz)	129.8	7.38 (2H, d, *J* = 8 Hz)	128.6
3,5	6.73 (2H, d, *J* = 8.8 Hz)	115.5	6.82 (2H, d, *J* = 8 Hz)	114.8
4		159.9		158.6
7	6.20 (1H, d, *J* = 15.9)	114.3	6.21 (1H, d, *J* =16 Hz)	114.0
8	7.52 (1H, d, *J* = 15.9 Hz)	145.3	7.54 (1H, d, *J* =16 Hz)	143.4
COOH		169.7		167.7

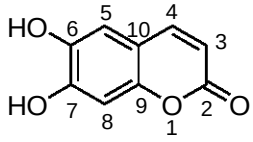
Scheme 3.2 describes the complete isolation of compounds **79**, **160** and **164**-**165** from the aerial parts of *G. divaricata*.

Scheme 3.2 The complete isolation of compounds **79**, **160** and **164**-**165** from *G. divaricata*



3.3.3 Coumarin compound from the aerial part of *G. divaricata*

3.3.3.1 Coumarin compound: esculetin **166**



166

Compound **166** was obtained as yellowish crystals. The ESI Mass spectrum of compound **166** displayed an ion peak at m/z 177 $[M-H]^-$. The ^1H NMR spectrum of compound **166** displayed two characteristic doublets at 7.70 (1H, d, *J* = 9.9 Hz, H-4) and 6.09 (1H, d, *J* = 9.9 Hz, H-3). Combined with the carbon signal at 144.7 (C-4) and 111.5 (C-3) in its ^{13}C NMR spectrum, the presence of a coumarin skeleton was deduced. Besides, the two singlet proton signals at 6.85 and 6.67, indicated a 6,7-disubstituted B-ring. Additionally, the chemical shifts in the NMR spectra were in close agreement to those observed for esculetin in literature data.[180]

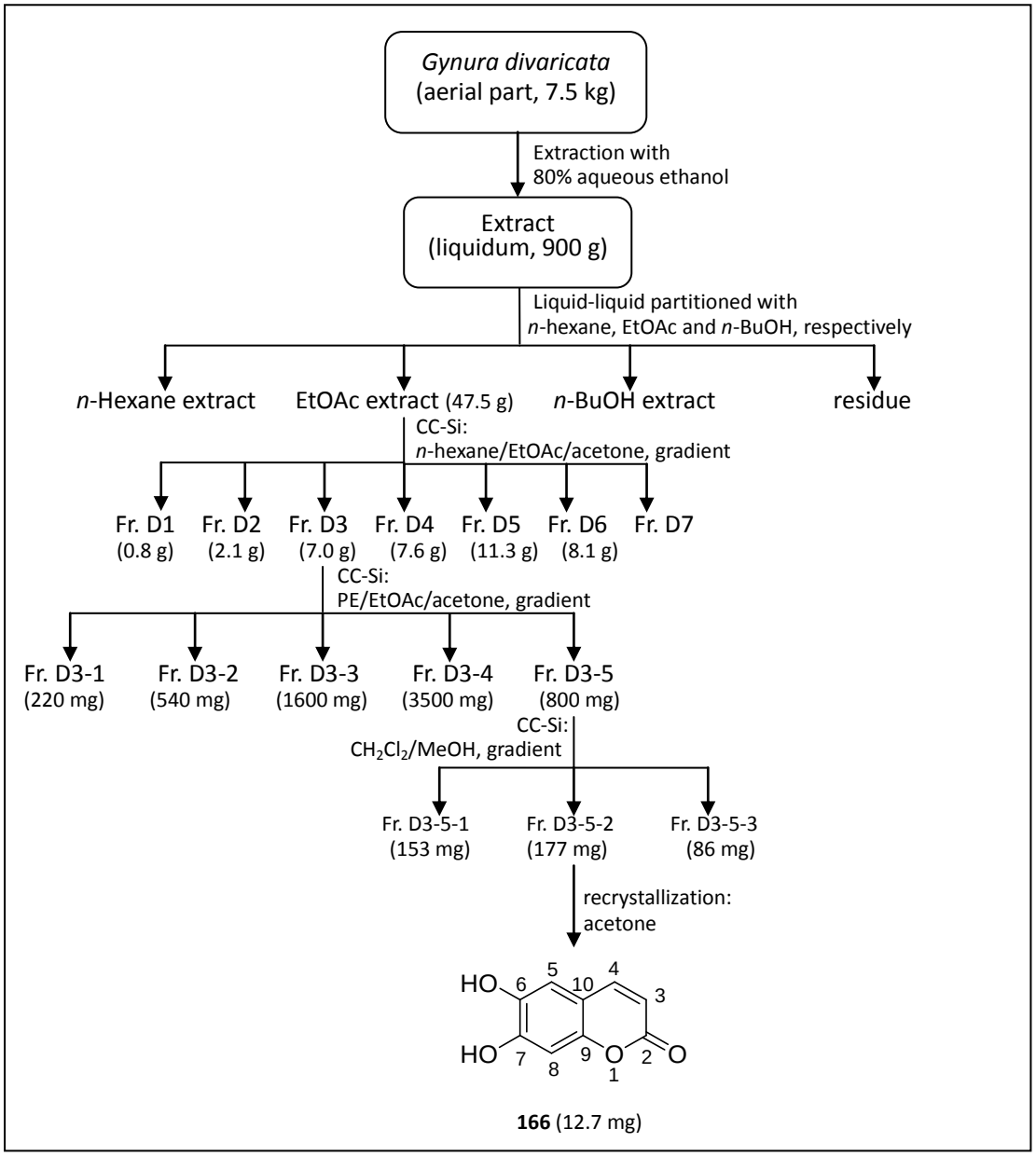
The comparison of characteristic NMR data of compound **166** with literature data is listed in Table 3.36.[180]

Table 3.36 Comparison of characteristic ^1H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of esculetin **166** with literature data (400/100 MHz, DMSO-d_6)[180]

Position	This work		literature data	
	^1H	^{13}C	^1H	^{13}C
1				
2		163.0		160.9
3	6.09 (1H, d, *J* = 9.9 Hz)	111.5	6.15 (1H, d, *J* = 9.6 Hz)	111.7
4	7.70 (1H, d, *J* = 9.9 Hz)	144.7	7.85 (1H, d, *J* = 9.6 Hz)	144.5
5	6.85 (1H, s)	111.7	6.97 (1H, s)	112.5
6		143.2		142.9
7		150.7		150.5
8	6.67 (1H, s)	102.3	6.74 (1H, s)	102.8
9		149.1		148.7
10		111.2		110.9

Scheme 3.3 describes the complete isolation of compound **166** from the aerial parts of *G. divaricata*.

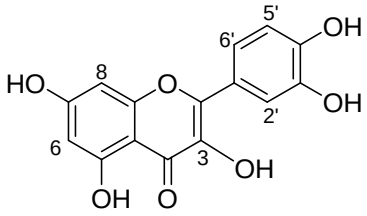
Scheme 3.3 The complete isolation of compound **166** from *G. divaricata*



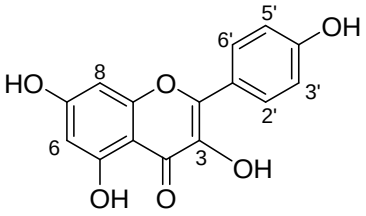
166 (12.7 mg)

3.3.4 Flavonoids from the aerial part of *G. divaricata*

3.3.4.1 Flavonol compounds: quercetin **35** and kaempferol **40**



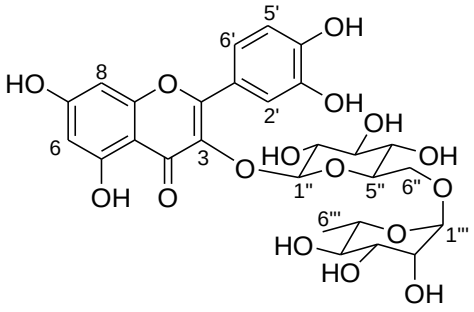
35



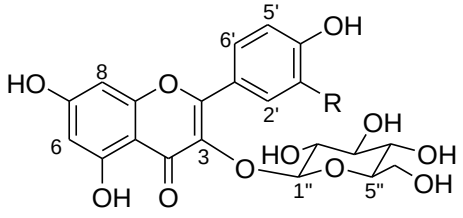
40

Compound **35** and **40** were both obtained as a yellow amorphous powder. Their ESI mass spectra displayed quasi-molecular ion peaks $[M-H]^-$ at *m/z* 301 and $[M+H]^+$ at m/z 287, compatible with their molecular formula $C_{15}H_{10}O_7$ and $C_{15}H_{10}O_6$, respectively. In their ^1H NMR spectra, both compounds displayed two doublets around δ 6.08 and 6.30 with a coupling constant of about 2 Hz, which could be assigned to the meta-coupled protons on the A-ring of a flavonoid. In the ^1H NMR spectrum of compound **35**, the aromatic region also exhibited an ABX system at δ 7.63 (1H, d, *J* = 2.1 Hz, H-2′), 7.53 (1H, dd, *J* = 8.1, 2.1 Hz, H-6′) and 6.78 (1H, d, *J* = 8.1 Hz, H-5′) due to a 3′,4′-disubstituted B-ring. On the other hand, a typical A_2B_2 system was observed in the ^1H NMR spectrum of compound **40** at δ 7.99 (2H, d, *J* = 9.1 Hz, H-2′, 6′) and 6.81 (2H, d, *J* = 9.1 Hz, H-3′, 5′), which were assignable to the protons on a para-substituted B-ring. The ^{13}C NMR spectra of **35** and **40** both showed the presence of fifteen aromatic carbon signals. Based on the NMR data and comparison of the data given in the literatures,[182,183] the structures of compound **35** and **40** were identified as quercetin and kaempferol, respectively.

3.3.4.2 Flavonol glycoside compounds: rutin **38**, quercetin 3-O-β-D-glucoside **36** and kaempferol 3-O-β-D-glucoside **41**



38



36: R = OH

41: R = H

Compounds **38** and **36** were isolated as a yellow amorphous powder. Their negative ESI mass spectra gave quasi-molecular ion peaks $[M-H]^-$ at m/z 609 and 463, suggesting their molecular formula $C_{27}H_{30}O_{16}$ and $C_{21}H_{20}O_{12}$, respectively. Their ^1H NMR spectra looked very similar in the aromatic region, indicating the presence of a quercetin moiety in their structures. Sugar residues were also observed in ^1H NMR spectra and their integration indicated that compound **36** contained one simple sugar while compound **38** contained two. By comparison of ^{13}C NMR data with literature data, the sugar moiety of compound **36** was identified as glucose, while compound **38** had glucose and rhamnose parts. The anomeric proton signals appeared as doublets at δ 5.01 and 5.13 with a coupling constant for both of 7.2 Hz suggesting the presence of β-glucopyranose units in compound **38** and **36** respectively. In the HMBC spectrum of compound **36**, a crosspeak between C-3 at δ 135.6 and H-1″ at δ 5.13 established the linkage point of quercetin and β-glucopyranose. Based on these facts, the structure of compound **36** was identified as quercetin 3-O-β-D-glucopyranoside (isoquercitrin). Finally, by comparison of the ^{13}C NMR data of the sugar moiety with literature data,[181] compound **38** was characterized as quercetin 3-O-rutinoside (rutin).

Compound **41** was obtained as a yellow amorphous powder. The ESI Mass spectrum displayed an ion peak at m/z 447 $[M-H]^-$, suggesting its molecular formula as $C_{21}H_{20}O_{11}$. The aromatic protons were present at δ 6.25 and 6.07 as meta-related doublets (*J* = 1.8 Hz) for H-8 and H-6, and at δ 7.96 (d) and 6.79 (d) integrating for two protons each with a coupling constant of 8.8 Hz for H-2′, 6′ and H-3′, 5′, respectively. The signals in the aromatic region indicated the presence of a kaempferol aglycone. The sugar part was confirmed by a comparison of the TLC Rf value and the HPLC retention time with those of standards. Thus, compound **41** was characterized as kaempferol 3-O-β-D-glucoside (astragalin).

The spectral data of the compounds isolated in the present study match well with those reported earlier. The comparison of characteristic NMR data of compounds **35**-**36**, **38** and **40-41** with literature data is listed in the Table 3.37 and 3.38.[181,182,183]

Table 3.37 Comparison of characteristic ^{1}H NMR (300 MHz, MeOH-d_4) data of compounds **35**-**36**, **38** and **40**-**41** with literature data (400 MHz, MeOH-d_4)

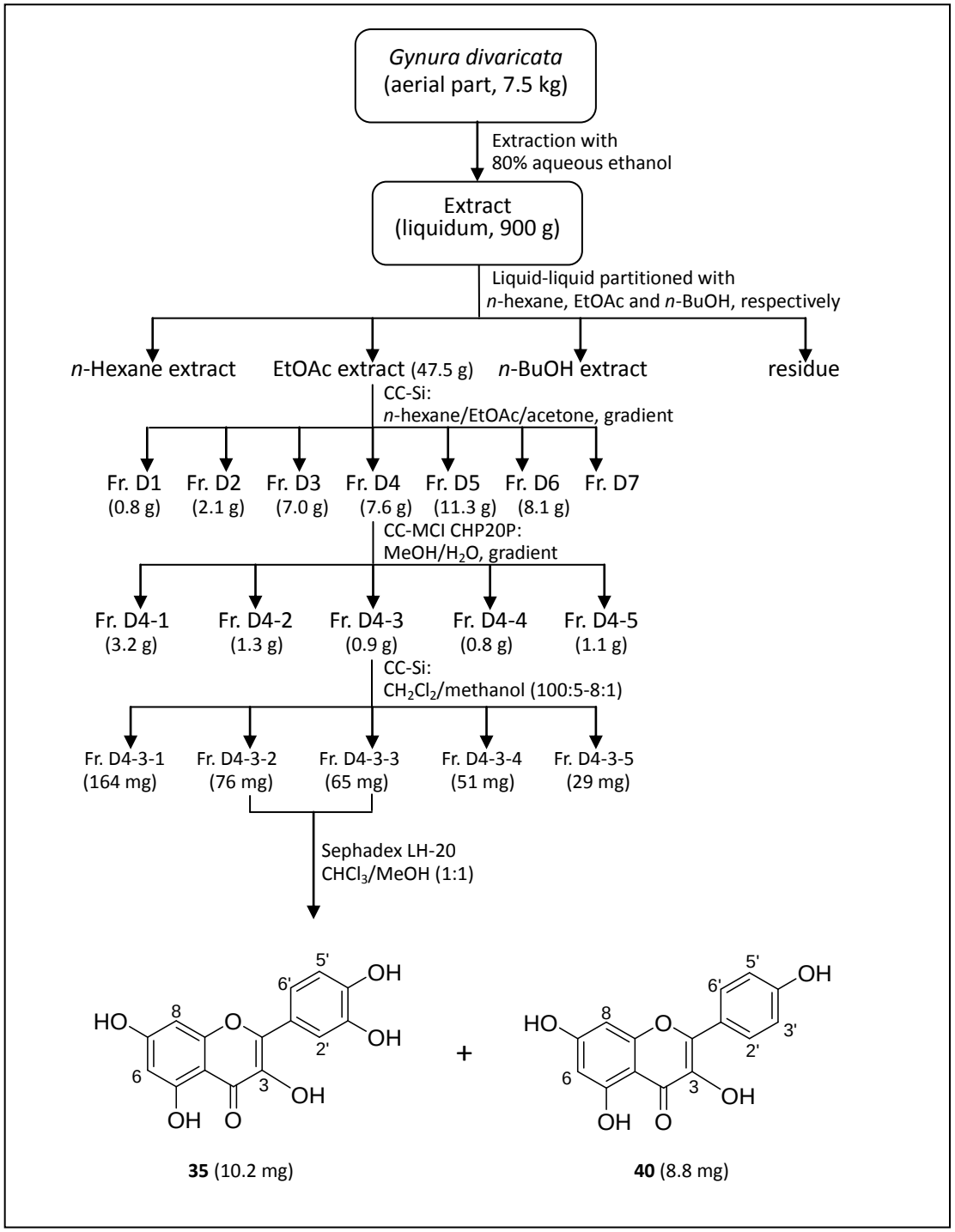
Position	This work				
	38	**36**	**41**	**35**	**40**
H-6	6.12 (1H, s)	6.08 (1H, d, *J* = 1.9 Hz)	6.07 (1H, d, *J* = 1.8 Hz)	6.08 (1H, d, *J* = 1.8 Hz)	6.08 (1H, d, *J* = 2.1 Hz)
H-8	6.31 (1H, s)	6.27 (1H, d, *J* = 1.9 Hz)	6.25 (1H, d, *J* = 1.8 Hz)	6.29 (1H, d, *J* = 1.8 Hz)	6.30 (1H, d, *J* = 2.1 Hz)
H-2′	7.57 (1H, d, *J* = 2.2 Hz)	7.60 (1H, d, *J* = 2.2 Hz)	7.96 (1H, d, *J* = 8.8 Hz)	7.63 (1H, d, *J* = 2.1 Hz)	7.99 (1H, d, *J* = 9.1 Hz)
H-3′			6.79 (1H, d, *J* = 8.8 Hz)		6.81 (1H, d, *J* = 9.1 Hz)
H-5′	6.78 (1H, d, *J* = 8.7 Hz)	6.77 (1H, d, *J* = 8.8 Hz)	6.79 (1H, d, *J* = 8.8 Hz)	6.78 (1H, d, *J* = 8.1 Hz)	6.81 (1H, d, *J* = 9.1 Hz)
H-6′	7.54 (1H, d, *J* = 8.7, 2.2 Hz)	7.49 (1H, dd, *J* = 8.8, 2.2 Hz)	7.96 (1H, d, *J* = 8.8 Hz)	7.53 (1H, dd, *J* = 8.1, 2.1 Hz)	7.99 (1H, d, *J* = 9.1 Hz)
H-1″	5.01 (1H, d, *J* = 7.2 Hz)	5.13 (1H, d, *J* = 7.2 Hz)	5.12 (1H, d, *J* = 6.9 Hz)		
H-1‴	4.42 (1H, brs)				
H-sugar	3.26-3.73 (11H, m)	3.30-3.64 (6H, m)	3.30-3.62 (6H, m)		
CH_3-6‴	1.02 (3H, d, *J* = 5.4 Hz)				
Position	Literature data				
	rutin[181]	isoquercitrin[182]	astragalin[183]	quercetin[183]	kaempferol[182]
H-6	6.20 (1H, d, *J* = 1.8 Hz)	6.16 (1H, d, *J* = 2.0 Hz)	6.19 (1H, brs)	6.17 (1H, d, *J* = 2.0 Hz)	6.16 (1H, d, *J* = 2.0 Hz)
H-8	6.39 (1H, d, *J* = 2.2 Hz)	6.34 (1H, d, *J* = 2.0 Hz	6.38 (1H, brs)	6.37 (1H, d, *J* = 2.0 Hz)	6.36 (1H, d, *J* = 2.0 Hz)
H-2′	7.66 (1H, d, *J* = 1.8 Hz)	7.70 (1H, d, *J* = 2.4 Hz)	8.04 (1H, d, *J* = 8.4 Hz)	7.73 (1H, d, *J* = 2.0 Hz)	8.06 (1H, d, *J* = 9.2 Hz)
H-3′			6.87 (1H, d, *J* = 8.4 Hz)		6.89 (1H, d, *J* = 9.2 Hz)
H-5′	6.86 (1H, d, *J* = 8.0 Hz)	6.85 (1H, d, *J* = 8.6 Hz)	6.87 (1H, d, *J* = 8.4 Hz)	6.87 (1H, d, *J* = 8.0 Hz)	6.89 (1H, d, *J* = 9.2 Hz)
H-6′	7.60 (1H, dd, *J* = 8.0, 1.8 Hz)	7.55 (1H, dd, *J* = 8.6, 2.4 Hz)	8.04 (1H, d, *J* = 8.4 Hz)	7.62 (1H, dd, *J* = 7.5, 2.0 Hz)	8.06 (1H, d, *J* = 9.2 Hz)
H-1″	5.09 (1H, d, *J* = 7.8 Hz)	5.22 (1H, d, *J* = 7.2 Hz)	5.23 (1H, d, *J* = 7.2 Hz)		
H-1‴	4.51 (1H, d, *J* = 1.8 Hz)				
H-sugar	3.25-3.80 (11H, m)	3.22-3.71 (6H, m)	3.18-3.71 (6H, m)		
CH_3-6‴	1.11 (3H, d, *J* = 6.0 Hz)				

Table 3.38 Comparison of characteristic ^{13}C NMR (75 MHz, MeOH-d_4) data of compounds **35**-**36**, **38** and **40-41** with literature data (100 MHz, MeOH-d_4)

Position	**38**	rutin[181]	**36**	isoquercitrin[182]	**35**	quercetin[183]	**40**	kaempferol[182]
2	158.0	158.5	158.5	158.1	146.7	147.9	146.6	147.8
3	134.3	135.6	135.6	135.4	135.9	137.2	135.9	137.1
4	178.1	179.4	179.5	179.1	176.0	177.3	175.8	177.1
5	161.7	162.5	163.1	162.7	156.9	162.5	156.9	162.3
6	98.6	99.9	100.0	99.7	97.9	99.3	97.9	99.2
7	164.7	166.0	166.5	165.7	164.2	165.7	164.3	165.3
8	93.5	94.8	94.8	94.6	93.1	94.4	93.1	94.4
9	157.2	159.3	159.0	158.2	161.2	158.2	161.2	158.1
10	104.3	105.6	105.6	105.4	103.2	104.4	103.2	104.4
1’	121.8	123.1	123.2	122.6	122.8	124.1	122.2	123.6
2’	114.7	117.6	116.0	115.8	114.7	116.0	129.4	130.5
3’	144.5	145.8	147.0	145.5	144.9	146.2	115.0	116.2
4’	148.5	149.7	149.9	149.6	147.4	148.7	159.1	160.3
5’	116.4	116.1	117.5	117.4	114.9	116.2	115.0	116.2
6’	122.2	123.5	123.1	123.0	120.3	121.6	129.4	130.5
1”	103.4	104.7	104.3	104.3				
2”	74.4	75.7	75.7	75.6				
3”	75.9	77.2	78.1	78.2				
4”	70.9	71.4	71.2	71.1				
5”	76.9	78.1	78.4	78.0				
6”	67.2	68.6	62.6	62.5				
1”’	101.1	102.4						
2”’	70.8	72.0						
3”’	70.1	72.2						
4”’	72.6	73.9						
5”’	68.4	69.7						
6”’	16.6	17.9						

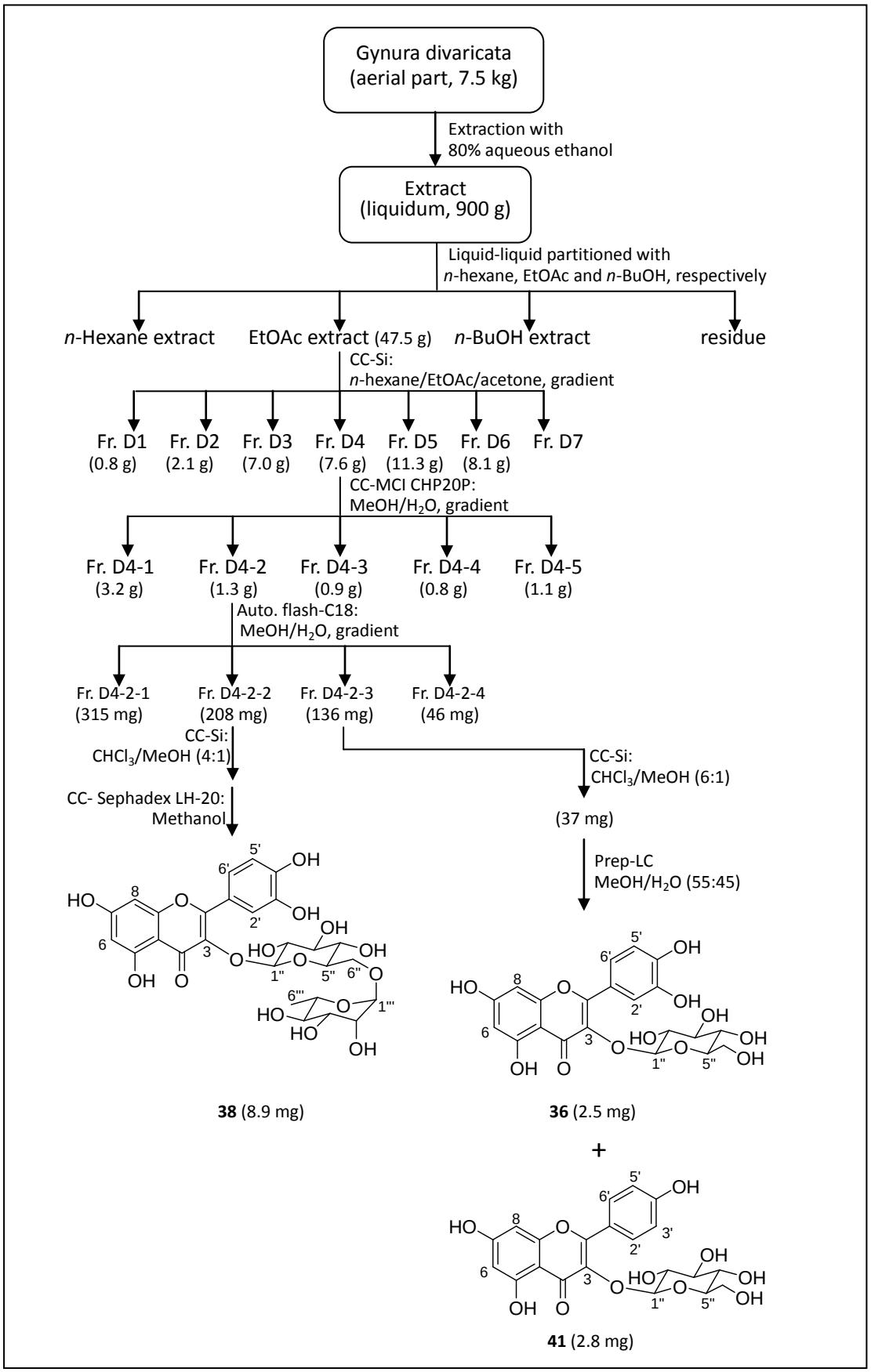
Scheme 3.4 describes the complete isolation of compounds **35** and **40** from the aerial parts of *G. divaricata*.

Scheme 3.4 The complete isolation of compounds **35** and **40** from *G. divaricata*



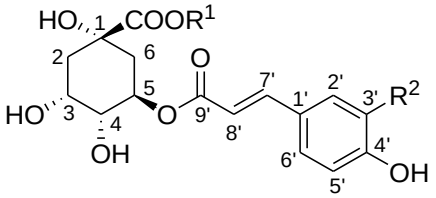
Scheme 3.5 describes the complete isolation of compounds **36**, **38** and **41** from the aerial parts of *G. divaricata*.

Scheme 3.5 The complete isolation of compounds **36**, **38** and **41** from *G. divaricata*



3.3.5 Hydroxycinnamic acid conjugates from the aerial part of *G. divaricata*

3.3.5.1 Caffeoylquinic acid derivatives: 5-O-caffeoylquinic acid **74**, methyl 5-O-caffeoylquinate **172**, 5-O-feruloylquinic acid **173** and 5-O-*p*-coumaroylquinic acid **171**



74: R^1 = H, R^2 = OH

172: R^1 = Me, R^2 = OH

173: R^1 = H, R^2 = OMe

171: R^1 = H, R^2 = H

Compound **74** was isolated as a white powder. The negative ESI mass spectrum of **74** displayed a pseudomolecular ion peak at 353 $[M-H]^-$, suggesting a molecular formula $C_{16}H_{18}O_9$. The ^{1}H NMR spectrum showed one ABX spin system at δ 7.04 (1H, d, *J* = 1.7 Hz), 6.95 (1H, d, *J* = 8.3 Hz) and 6.77 (1H, d, *J* = 8.3 Hz) in the aromatic region originating from a 1,3,4-trisubstituted benzene ring. A pair of doublets appeared at δ 7.56 and 6.27 with coupling constant 16.0 Hz assigned to a trans-configurated olefinic moiety conjugated to a carbonyl group. In the ^{13}C NMR spectrum, two methylenes at δ 38.8 and 38.2, three oxygen-bearing methines at δ 73.5, 72.0 and 71.3, one oxygen-bearing quaternary carbon at δ 76.1 and one carboxyl group at δ 177.0, revealed the presence of a quinic acid moiety. Besides, a caffeoyl moiety was observed as well. In the ^{1}H NMR spectrum, the downfield shift of the resonance signal of H-5 around at δ 5.29-5.36 indicated that the hydroxyl group on C-5 was acylated with a caffeic acid moiety.[193] Thus, compound **74** was identified as 5-O-caffeoylquinic acid. The spectral data of the isolated compound **74** in the present study match well with those reported earlier.[189]

Compound **172** was isolated as a white powder. The negative ESI mass spectrum of **172** displayed a pseudomolecular ion peak at 367 $[M-H]^-$, suggesting a molecular formula $C_{17}H_{20}O_9$. All of the present signals in the NMR spectra of compound **172** were similar as those of compound **74**. The difference was that the ^{1}H NMR spectrum of compound **172** showed a extra singlet at δ 3.69 integrating for three protons, along with the corresponding oxygen-bearing methyl observed at δ 53.0 in its ^{13}C NMR spectrum. By comparison of the chemical shift of this MeO group with data in the literature reported,[194] it was concluded that the methoxy group was part of the carboxylic unit. Based on this, compound **172** was identified as methyl 5-O-caffeoylquinate. The spectral data of the isolated compound **172** in the present study match well with those reported earlier.[189]

Compound **173** was isolated as a white powder. The negative ESI mass spectrum of **173** displayed a pseudomolecular ion peak at 367 $[M-H]^-$, suggesting a molecular formula $C_{17}H_{20}O_9$. All of the spectral data indicated that **173** was a methoxy-caffeoylquinic acid derivative with similar molecular structure as compound **172** (methyl 5-O-caffeoylquinate). In the ^1H NMR spectra of compound **173**, the downfield shift of OCH_3 (singlet, δ 3.89) in comparison to that of methyl 5-O-caffeoylquinate (singlet, δ 3.69), suggested that the methoxy was attached to the benzene ring.[193,194] By comparison of the chemical shift of compound **173** with literature reported,[190] the structure of compound **173** was assigned as 5-O-feruloylquinic acid.

Compound **171** was isolated as a white powder. The negative ESI mass spectrum of **171** displayed a pseudomolecular ion peak at 337 $[M-H]^-$, suggesting a molecular formula $C_{16}H_{18}O_8$. The appearance of its proton chemical shifts and proton-proton coupling patterns in the ^1H NMR spectrum were consistent with a p-coumaroyl chemical structure and a quinic acid ring system. The ^1H and ^{13}C NMR data of the quinic acid moiety were analogous to those of the above three compounds, and, therefore, compound **171** was assigned as 5-O-*p*-coumaroylquinic acid. The spectral data of the isolated compound **171** in the present study match well with those reported earlier.[188]

The comparison of characteristic NMR data of compounds **74** and **171-173** with literature data is listed in Table 3.39, 3.40, 3.41 and 3.42.[188,189,190]

Table 3.39 Comparison of characteristic ^1H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of 5-O-caffeoylquinic acid **74** with literature data (300/75 MHz, MeOH-d_4)[189]

Position	Compound **74**		literature data	
	^1H	^{13}C	^1H	^{13}C
1		76.1		76.1
2	2.01-2.08 (2H, m)	38.2	1.92-1.98 (2H, m)	38.2
3	4.12-4.20 (1H, m)	71.3	4.10 (1H, m)	71.3
4	3.70-3.75 (1H, m)	72.0	3.67 (1H, m)	72.0
5	5.29-5.36 (1H, m)	73.5	5.36 (1H, m)	73.5
6	2.16-2.21 (2H, m)	38.8	2.11 (2H, m)	38.8
1’		127.8		127.8
2’	7.04 (1H, d, *J* = 1.7 Hz)	115.3	7.04 (1H, d, *J* = 1.8 Hz)	115.2
3’		147.1		147.1
4’		149.6		149.6
5’	6.77 (1H, d, *J* = 8.3 Hz)	116.5	6.76 (1H, d, *J* = 8.2 Hz)	116.5
6’	6.95 (1H, d, *J* = 8.3 Hz)	123.0	6.94 (1H, d, *J* = 8.1, 1.8 Hz)	123.0
7’	7.56 (1H, d, *J* = 16.0 Hz)	146.8	7.56 (1H, d, *J* = 15.9 Hz)	146.8
8’	6.27 (1H, d, *J* = 16.0 Hz)	115.2	6.28 (1H, d, *J* = 15.9 Hz)	115.1
9’		168.7		168.7
COO		177.0		177.0

Table 3.40 Comparison of characteristic ^{1}H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of methyl 5-O-caffeoylquinate **172** with literature data (300/75 MHz, MeOH-d_4)[189]

Position	Compound **172**		literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		75.8		75.8
2	1.97-2.06 (2H, m)	37.7	1.96-2.02 (2H, m)	37.8
3	4.12-4.15 (1H, m)	70.3	4.12 (1H, m, H-3)	70.3
4	3.72-3.75 (1H, m)	72.1	3.72 (1H, m, H-4)	72.1
5	5.24-5.30 (1H, m)	72.5	5.26 (1H, m, H-5)	72.5
6	2.18-2.25 (2H, m)	38.0	2.17 (2H, m)	38.0
1’		127.6		127.6
2’	7.04 (1H, d, *J* = 2.2 Hz)	115.1	7.03 (1H, d, *J* = 1.8 Hz)	115.1
3’		147.2		147.2
4’		149.7		149.7
5’	6.78 (1H, d, *J* = 8.0 Hz)	116.5	6.77 (1H, d, *J* = 8.1 Hz)	116.5
6’	6.95 (1H, dd, *J* = 8.0, 2.2 Hz)	123.0	6.94 (1H, dd, *J* = 8.2, 1.8 Hz)	123.0
7’	7.52 (1H, d, *J* = 16.0 Hz)	146.9	7.51 (1H, d, *J* = 15.9 Hz)	146.9
8’	6.22 (1H, d, *J* = 16.0 Hz)	115.0	6.21 (1H, d, *J* = 15.9 Hz)	115.0
9’		168.3		168.3
COO		175.4		175.4
OCH_3	3.69 (3H, s)	53.0	3.68 (3H, s)	53.0

Table 3.41 Comparison of characteristic ^{1}H NMR (300 MHz, MeOH-d_4) data of 5-O-feruloylquinic acid **173** with literature data (360 MHz, MeOH-d_4)[190]

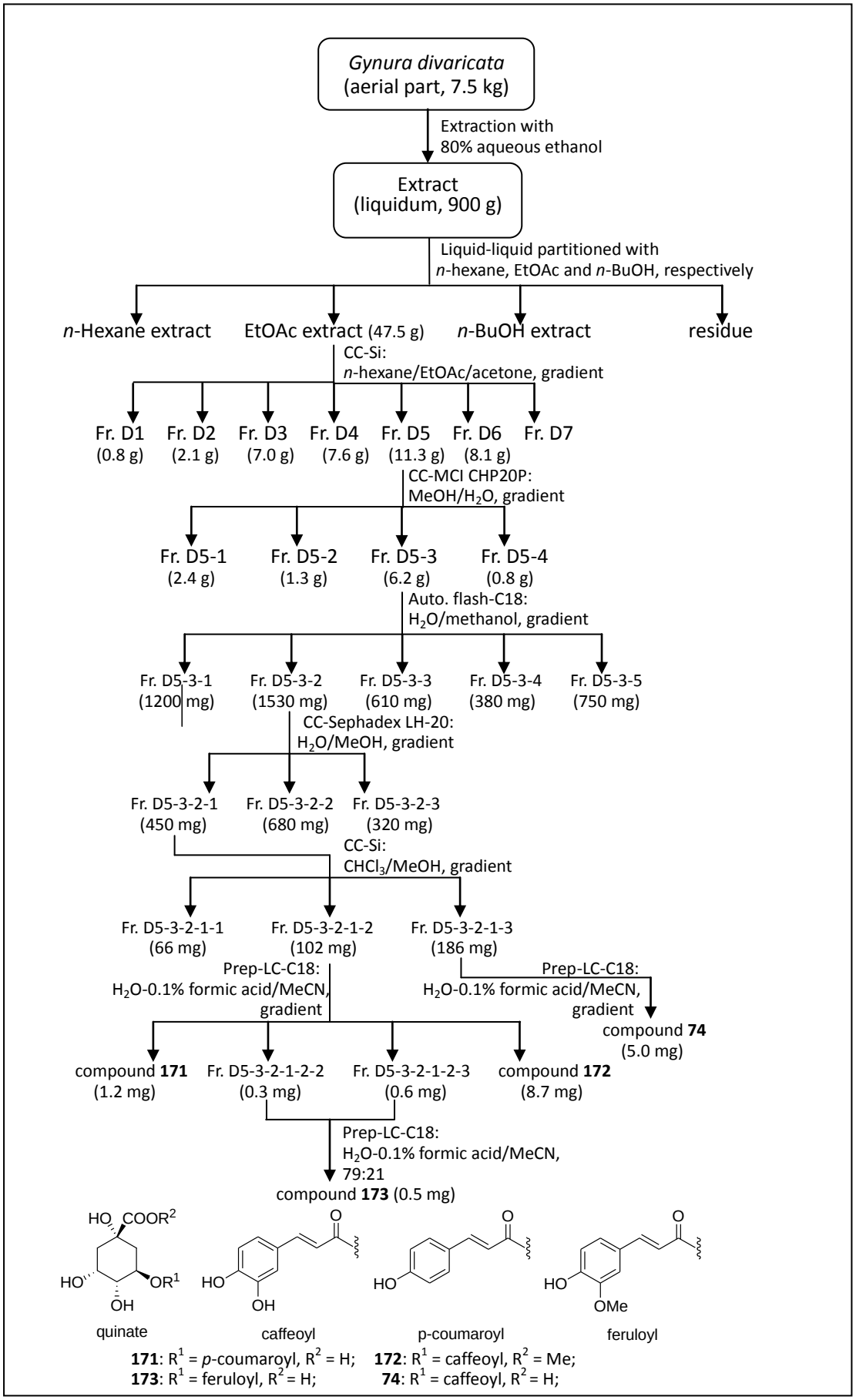
Position	Compound **173**	literature data
	^{1}H	^{1}H
1		
2	1.87-2.21 (2H, m)	2.19-1.92 (2H, m)
3	4.16-4.22 (1H, m)	4.15 (1H, q, *J* = 2.8 Hz)
4	3.69-3.77 (1H, m)	3.69 (1H, dd, *J* = 9.9, 3.1 Hz)
5	5.30-5.40 (1H, m)	5.38 (1H, ddd, *J* = 11.3, 10.1, 5.0 Hz)
6	1.87-2.21 (2H, m)	1.92-2.19 (2H, m)
1’		
2’	7.19 (1H, s)	7.15 (1H, d, *J* = 1.9 Hz)
3’		
4’		
5’	6.81 (1H, d, *J* = 7.4 Hz)	6.77 (1H, d, *J* = 8.2 Hz)
6’	7.08 (1H, d, *J* = 7.4 Hz)	7.04 (1H, dd, *J* = 8.3, 1.8 Hz)
7’	7.62 (1H, d, *J* = 16.0 Hz)	7.63 (1H, d, *J* = 15.9 Hz)
8’	6.36 (1H, d, *J* = 16.0 Hz)	6.35 (1H, d, *J* = 15.9 Hz)
9’		
OCH_3	3.89 (3H, s)	3.87 (3H, s)

Table 3.42 Comparison of characteristic ^{1}H NMR (300 MHz, MeOH-d_4) and ^{13}C NMR (75 MHz, MeOH-d_4) data of 5-O-*p*-coumaroylquinic acid **171** with literature data (300/75 MHz, MeOH-d_4)[188]

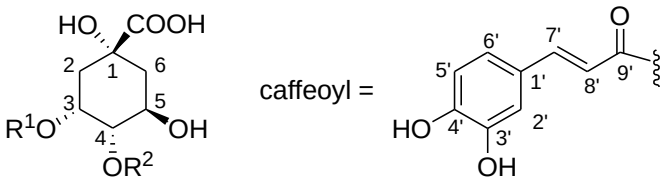
Position	Compound **171**		literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		76.0		76.60
2	2.01-2.25 (2H, m)	37.9	2.01-2.29 (2H, m)	38.56
3	4.12-4.20 (1H, m)	71.7	3.90 (1H, d, *J* = 3.1 Hz)	72.43
4	3.73 (1H, dd, *J* = 8.3, 2.8 Hz)	71.0	3.77 (1H, dd, *J* = 8.6, 3.0 Hz)	71.66
5	5.28-5.38 (m)	73.1	5.33 (1H, m)	73.82
6	2.01-2.25 (2H, m)	38.5	2.01-2.29 (2H, m)	39.14
1’		127.0		127.70
2’	7.47 (1H, d, *J* = 8.8 Hz)	130.9	7.48 (1H, d, *J* = 8.6Hz)	131.69
3’	6.81 (1H, d, *J* = 8.8 Hz)	116.5	6.84 (1H, d, *J* = 8.6 Hz)	117.31
4’		160.8		161.48
5’	6.81 (1H, d, *J* = 8.8 Hz)	116.5	6.84 (1H, d, *J* = 8.6 Hz)	117.31
6’	7.47 (1H, d, *J* = 8.8 Hz)	130.9	7.48 (1H, d, *J* = 8.6Hz)	131.69
7’	7.63 (1H, d, *J* = 16.0 Hz)	146.5	7.65 (1H, d, *J* = 16.0 Hz)	147.34
8’	6.33 (1H, d, *J* = 16.0 Hz)	115.0	6.34 (1H, d, *J* = 16.0Hz)	115.68
9’		168.5		169.38
COO		177.1		177.64

Scheme 3.6 describes the complete isolation of compounds **74** and **171-173** from the aerial parts of *G. divaricata*.

Scheme 3.6 The complete isolation of compounds **74** and **171-173** from *G. divaricata*



3.3.5.2 Dicaffeoylquinic acid derivatives: 3,4-dicaffeoylquinic acid **167**, 3,5-dicaffeoylquinic acid **72**, 4,5-dicaffeoylquinic acid **73**, methyl 3,4-dicaffeoylquinate **168**, methyl 3,5-dicaffeoylquinate **169**,methyl 4,5-dicaffeoylquinate **170** and ethyl 4,5-dicaffeoylquinate **174**



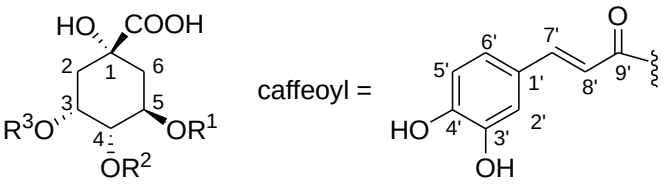
167: $R^1 = R^2$ = caffeoyl

Compound **167** was isolated as a pale green powder. The negative ESI mass spectrum of **167** displayed a pseudomolecular ion peak at 515 $[M-H]^-$, suggesting a molecular formula $C_{25}H_{24}O_{12}$. The ^{1}H NMR spectrum of **167** displayed two sets of proton signals with two pairs of doublets with a coupling constant of 16 Hz at δ 7.57, 7.55, and 6.29, 6.26, due to two (*E*)-olefinic moieties each attached to a carbonyl group, and a pair of ABX spin-spin coupling systems at δ (7.04, 7.02, d, 2 Hz), (6.93, 6.88, dd, 8, 2 Hz) and (6.77, 6.73, d, 8 Hz), respectively, due to two trisubstituted aromatic moieties. Thus, the presence of two caffeoyl moieties was deduced to be part of the chemical structure of **167**. In addition, one quinic acid moiety was observed due to three O-bearing methines at δ ca. 5.63 (m), 5.00 (dd) and 4.37 (m), along with a system integrating for four protons appearing at δ 1.99-2.38. The presence of one quinic acid moiety was further supported by the ^{13}C NMR spectrum of compound **167**. The position on which the two caffeoyl residues were present on the quinic acid moiety were deduced from the comparative analysis of ^{1}H NMR chemical shifts of the protons in 3-O-caffeoylquinic acid.[189] In compound **167**, the signals of H-3, H-4 and H-5 were shifted downfield by ca. 0.21, 1.28 and 0.35 ppm, respectively, compared to that of 3-O-caffeoylquinic acid. On the basis of paramagnetic chemical shifts due to acylation,[195] it was concluded that the hydroxyl group at C-4 of compound **167** was esterified by caffeic acid and it was identified as 3,4-dicaffeoylquinic acid. The spectral data of the isolated compound **167** in the present study match well with those reported earlier.[184,185]

The comparison of characteristic NMR data of compound **167** with literature data is listed in Table 3.43.[184,185]

Table 3.43 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of 3,4-dicaffeoylquinic acid **167** with literature data (400/125 MHz, CD_3OD)

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		75.6		73.79
2	1.99-2.38 (2H, m)	42.0	2.11-2.28 (2H, m)	40.57
3	5.60-5.67 (1H, m)	70.4	5.63 (1H, m)	68.72
4	5.00 (1H, dd, *J* = 3.3, 9 Hz)	76.6	5.12 (1H, br. d, *J* = 6.5 Hz)	75.29
5	4.31-4.41 (1H, m)	66.1	4.38 (1H, m)	64.37
6	1.99-2.38 (2H, m)	37.2	2.11-2.28 (2H, m)	35.63
COOH		178.5		176.62
1'/1"		128.0		126.36
2'/2"	7.04/7.02 (2H, d, *J* = 2 Hz)	115.4	7.02/7.01 (1H, d, *J* = 2.0 Hz)	113.82
3'/3"		146.9		145.41
4'/4"		149.7		148.22
5'/5"	6.77/6.73 (2H, d, *J* = 8 Hz)	116.7	6.76/6.75 (1H, d, *J* = 8.5 Hz)	115.06
6'/6"	6.93/6.88 (2H, dd, *J* = 8, 2 Hz)	123.5	6.92/6.91 (1H, dd, *J* = 8.5, 2.0 Hz)	121.82
7'/7"	7.57/7.55 (2H, d, *J* = 16 Hz)	147.6	7.60/7.52 (1H, d, *J* = 15.8 Hz)	145.97
8'/8"	6.29/6.26 (2H, d, *J* = 16 Hz)	115.2	6.29/6.19 (1H, d, *J* = 15.8 Hz)	113.49
9'/9"		168.9		167.20



72: $R^1 = R^3$ = caffeoyl, R^2 = H

73: $R^1 = R^2$ = caffeoyl, R^3 = H

Compound **72** and **73** were both isolated as a pale green powder. Their ESI and NMR spectral data indicated that compound **72** and **73** were isomers of compound **167** with the same molecular formula $C_{25}H_{24}O_{12}$. The observed chemical shifts of H-3, H-4 and H-5 were used to distinguish these three compounds from one to another. By comparison the spectral data of compound **72** with that of 5-O-caffeoylquinic acid,[189] a downfield shift by ca. 1.31 ppm was observed for H-3 in the quinic acid moiety, whereas H-4 and H-5 were only slightly affected. On the basis of paramagnetic chemical shifts due to acylation,[195] the structure of compound **72** as 3,5-dicaffeoylquinic acid was concluded. In the same manner, compound **73** was identified as 4,5-dicaffeoylquinic acid. The NMR spectral data of the isolated compounds in the present study match well with those reported earlier.[184,185,186]

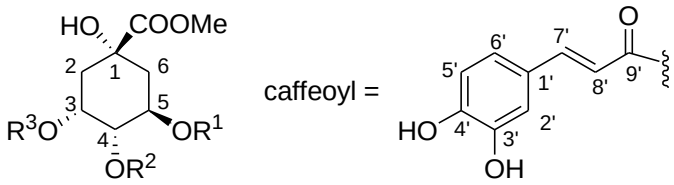
The comparison of characteristic NMR data of compounds **72** and **73** with literature data is listed in Table 3.44 and 3.45, respectively.[184,185,186]

Table 3.44 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of 3,5-dicaffeoylquinic acid **72** with literature data (400/125 MHz, CD_3OD)

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		74.8		73.34
2	2.04-2.34 (2H, m)	36.1	2.16-2.32 (2H, m)	34.62
3	5.34-5.46 (1H, m)	72.6	5.43 (1H, m)	70.68
4	3.97 (1H, dd, *J* = 7.5, 2.7 Hz)	70.7	3.97 (1H, dd, *J* = 7.5, 3.0 Hz)	69.27
5	5.34-5.46 (1H, m)	72.1	5.38 (1H, m, H-5)	71.15
6	2.04-2.34 (2H, m)	37.7	2.21-2.24 (2H, m)	36.29
COOH		177.6		176.09
1’/1”		127.9/127.8		126.50
2’/2”	7.07 (2H, s)	115.6/115.2	7.06 (2H, s)	113.80
3’/3”		146.8		145.38
4’/4”		149.6/149.5		148.20
5’/5”	6.79/6.77 (2H, d, *J* = 8.3 Hz)	116.4	6.78 (2H, d, *J* = 8.0 Hz)	115.05
6’/6”	6.97/6.96 (2H, dd, *J* = 8.3, 2.2 Hz)	123.1/123.0	6.96/6.97 (1H, dd, *J* = 8.0, 2.0 Hz)	121.66
7’/7”	7.62/7.59 (2H, d, *J* = 16 Hz)	147.3/147.0	7.62/7.58 (1H, d, *J* = 16.0 Hz)	145.86/145.63
8’/8”	6.36/6.27 (2H, d, *J* = 16 Hz)	115.1	6.35/6.26 (1H, d, *J* = 16.0 Hz)	114.17/113.68
9’/9”		168.9/168.4		167.47/166.95

Table 3.45 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of 4,5-dicaffeoylquinic acid **73** with literature data (400/125 MHz, CD_3OD)

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		75.8		74.31
2	1.93-2.38 (2H, m)	38.3	2.09 (2H, m)	37.02
3	4.27-4.42 (1H, m)	69.4	4.35 (1H, br s)	67.94
4	5.00-5.20 (1H, m)	76.2	5.10 (1H, dd, *J* = 2.8, 8.8 Hz)	74.36
5	5.50-5.68 (1H, m)	69.0	5.59 (1H, m)	67.59
6	1.93-2.38 (2H, m)	39.4	2.20 (2H, m)	37.94
COOH		177.1		175.45
1’/1”		127.6		126.28
2’/2”	6.96 (2H, s)	115.2	7.01/6.99 (2H, d, *J* = 2.0 Hz)	113.74
3’/3”		146.6		145.36
4’/4”		149.6		148.27
5’/5”	6.72/6.69 (2H, d, *J* = 7.2 Hz)	116.4	6.75/6.73 (2H, d, *J* = 8 Hz)	115.05
6’/6”	6.86/6.84 (2H, d, *J* = 7.2 Hz)	123.1	6.91/6.88 (2H, dd, *J* = 1.6, 6.8 Hz)	121.74
7’/7”	7.56/7.47 (2H, d, *J* = 16 Hz)	147.7/147.5	7.57/7.49 (2H, d, *J* = 16.0 Hz)	146.92/146.17
8’/8”	6.25/6.15 (2H, d, *J* = 16 Hz)	114.7	6.26/6.16 (2H, d, *J* = 16 Hz)	113.32/113.26
9’/9”		168.5/168.3		167.13/166.80



168: R^1 = H, R^2 = R^3 = caffeoyl
169: R^1 = R^3 = caffeoyl, R^2 = H
170: R^1 = R^2 = caffeoyl, R^3 = H

Compounds **168-170** were isolated as pale yellow powders. All their negative ESI mass spectra displayed the same pseudomolecular ion peaks at 529 $[M-H]^-$, suggesting their molecular formula $C_{26}H_{26}O_{12}$. All of the NMR spectral data indicated that compound **168-170** were methyl ester isomers of the above three dicaffeoylquinic acids. By comparison of the chemical shifts of the MeO group with literature reported,[194] it was concluded that the methoxy was attached to the carboxylic acid group. Thus, compounds **168-170** were identified as methyl 3,4-dicaffeoylquinate, methyl 3,5-dicaffeoylquinate and methyl 4,5-dicaffeoylquinate, respectively. The NMR spectral data of the isolated compounds in the present study match well with those reported earlier.[185,187]

The comparison of characteristic NMR data of compounds **168**-**170** with literature data is listed in Table 3.46, 3.47 and 3.48, respectively.[185,187]

Table 3.46 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of methyl 3,4-dicaffeoylquinate **168** with literature data (400/125 MHz, CD_3OD)

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		75.2		74.9
2	1.96-2.35 (2H, m)	41.3	2.03-2.27 (2H, dd)	38.5
3	5.52-5.66 (1H, m)	69.8	5.48 (1H, m)	69.1
4	4.95-5.15 (1H, m)	75.6	5.05 (1H, dd, *J* = 8.0, 3.2 Hz)	75.8
5	4.20-4.35 (1H, m)	69.8	4.29 (1H, dd, *J* = 3.2, 2.8 Hz)	68.6
6	1.96-2.35 (2H, m)	36.8	2.20 (2H, m)	38.4
COOC$\underline{H}_3$	3.73 (3H, s)	52.9	3.66 (3H, s)	53.1
$\underline{C}$OO		176.1		175.2
1’/1”		127.7		127.7/127.5
2’/2”	7.01 (2H, s)	115.1	6.97/6.95 (2H, s)	116.5
3’/3”		146.8		146.8
4’/4”		149.6		149.8/149.7
5’/5”	6.74 (2H, d, *J* = 7.7 Hz)	116.4	6.70 (2H, d, *J* = 8 Hz)	115.2/115.1
6’/6”	6.87 (2H, d, *J* = 7.7 Hz)	123.2/123.1	6.87 (2H, d, *J* = 6.4 Hz)	123.1
7’/7”	7.55/7.50 (2H, d, *J* = 16 Hz)	147.3	7.54/7.45 (2H, d, *J* = 16 Hz)	147.3
8’/8”	6.27/6.22 (2H, d, *J* = 16 Hz)	115.0/114.8	6.24/6.11 (2H, d, *J* = 16 Hz)	114.7/114.5
9’/9”		168.5		168.5/167.9

Table 3.47 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of methyl 3,5-dicaffeoylquinate **169** with literature data (400/125 MHz, CD_3OD)

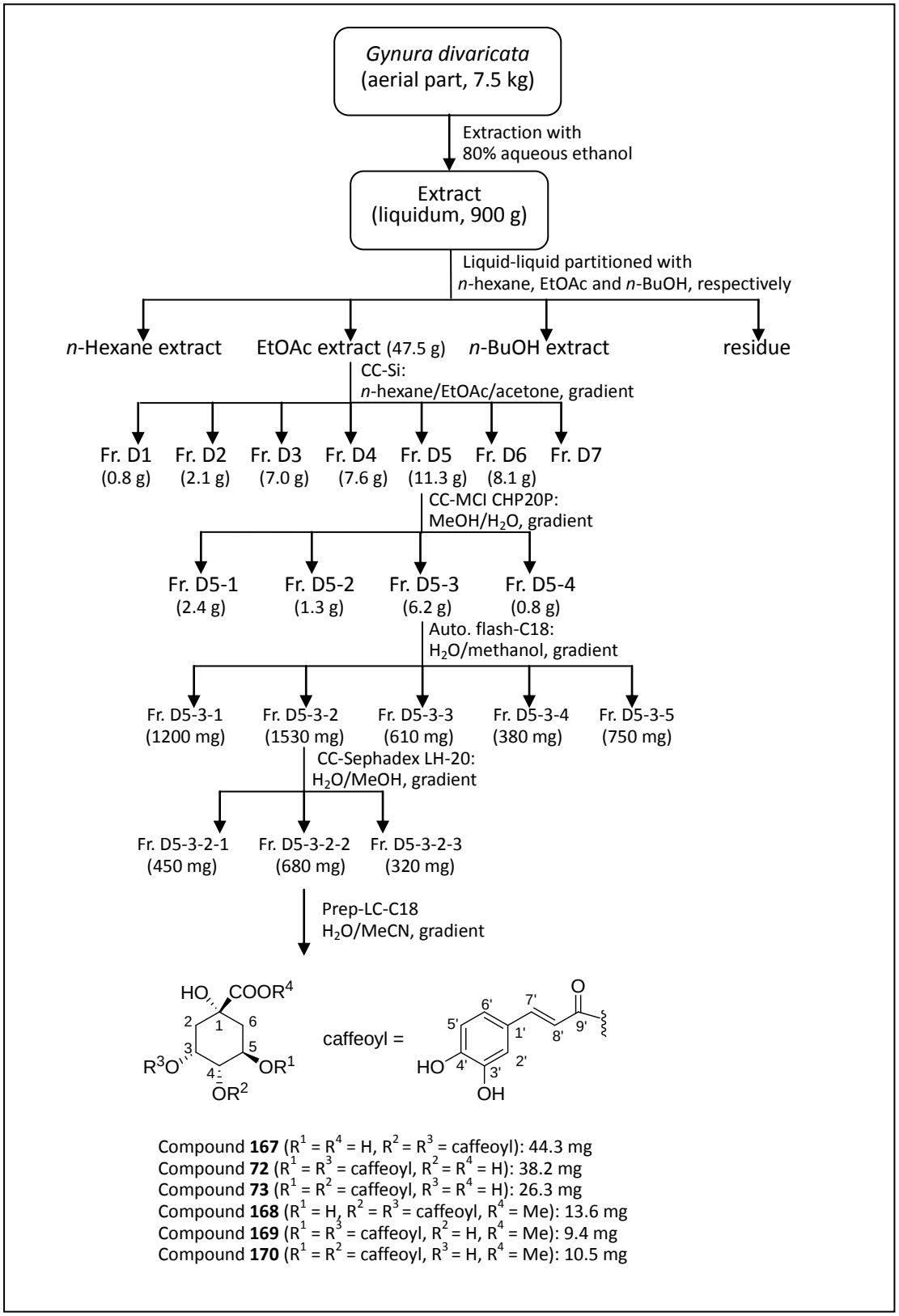
Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		74.6		73.20
2	2.11-2.36 (2H, m)	35.6	2.21-2.36 (2H, m)	35.35
3	5.27-5.35 (1H, m),	72.2	5.38 (1H, m)	70.79
4	3.98 (1H, dd, *J* = 7.5, 2.7 Hz)	69.7	3.99 (1H)	68.35
5	5.36-5.43 (1H, m)	72.0	5.40 (1H)	70.53
6	2.11-2.36 (2H, m)	37.0	2.21-2.36 (2H, m)	34.22
$COO\underline{C}H_3$	3.69 (3H, s)	53.0	3.70 (3H, s)	51.61
$\underline{C}OO$		175.6		174.20
1’/1”		127.9/127.6		126.44
2’/2”	7.07 (2H, d, *J* = 2 Hz)	115.5/115.1	7.07 (2H, d, *J* = 2.3 Hz)	113.69
3’/3”		146.9/146.8		145.48
4’/4”		149.8/149.6		148.37
5’/5”	6.80/6.77 (2H, d, *J* = 8 Hz)	116.6/116.5	6.80 (2H, d, *J* = 8.3 Hz)	115.13
6’/6”	6.99/6.96 (2H, d, *J* = 8 Hz)	123.1/123.0	6.99 (2H, d, *J* = 2.3, 8.3 Hz)	121.67
7’/7”	7.62/7.55 (2H, d, *J* = 16 Hz)	147.4/147.1	7.64/7.57 (2H, d, *J* = 16 Hz)	146.06/145.74
8’/8”	6.35/6.22 (2H, d, *J* = 16 Hz)	115.1/114.8	6.36/6.24 (2H, d, *J* = 16 Hz)	114.00/113.40
9’/9”		168.7/167.9		167.32/166.51

Table 3.48 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) NMR data of methyl 4,5-dicaffeoylquinate **170** with literature data (400/125 MHz, CD_3OD)

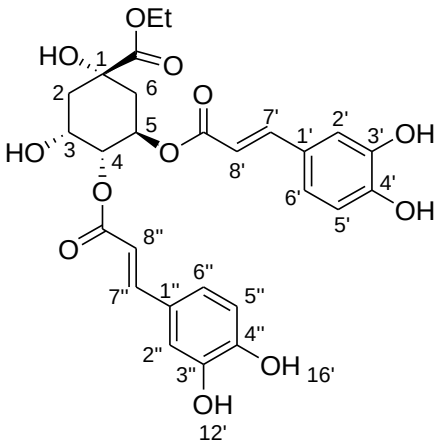
Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		74.8		74.04
2	1.99-2.36 (2H, m)	38.4	2.24-2.35 (2H, m)	36.96
3	4.30-4.39 (1H, m)	69.1	4.36 (1H, m)	67.13
4	5.08-5.15 (1H, m)	75.8	5.13 (1H, m)	74.32
5	5.48-5.59 (1H, m)	68.6	5.54 (1H, m)	67.66
6	1.99-2.36 (2H, m)	38.5	2.24-2.35 (2H, m)	36.96
$COO\underline{C}H_3$	3.72 (3H, s)	53.1	3.72 (3H, s)	51.68
$\underline{C}OO$		175.2		173.76
1’/1”		127.7/127.5		126.26/126.10
2’/2”	7.03 (2H, s)	115.1	7.04 (2H, d, *J* = 2.4 Hz)	113.73
3’/3”		146.8		145.42
4’/4”		149.8/149.7		148.38
5’/5”	6.78/6.75 (2H, d, *J* = 8 Hz)	116.5	6.77 (2H, d, *J* = 8.3 Hz)	115.09
6’/6”	6.91-6.93 (2H, m)	123.2	6.95 (2H, d, *J* = 2.3, 8.2 Hz)	121.75
7’/7”	7.61/7.51 (2H, d, *J* = 16 Hz)	147.7	7.62/7.52 (2H, d, *J* = 16 Hz)	146.31
8’/8”	6.30/6.18 (2H, d, *J* = 16 Hz)	114.7/114.5	6.32/6.19 (2H, d, *J* = 16 Hz)	113.28/113.10
9’/9”		168.5/167.9		167.05/166.46

Scheme 3.7 describes the complete isolation of compounds **72**-**73** and **167**-**170** from the aerial parts of *G. divaricata*.

Scheme 3.7 The complete isolation of compounds **72**-**73** and **167**-**170** from *G. divaricata*



Compound **167** (R^1 = R^4 = H, R^2 = R^3 = caffeoyl): 44.3 mg
Compound **72** (R^1 = R^3 = caffeoyl, R^2 = R^4 = H): 38.2 mg
Compound **73** (R^1 = R^2 = caffeoyl, R^3 = R^4 = H): 26.3 mg
Compound **168** (R^1 = H, R^2 = R^3 = caffeoyl, R^4 = Me): 13.6 mg
Compound **169** (R^1 = R^3 = caffeoyl, R^2 = H, R^4 = Me): 9.4 mg
Compound **170** (R^1 = R^2 = caffeoyl, R^3 = H, R^4 = Me): 10.5 mg



174

Compound **174** was isolated as a white powder. Its negative ESI mass spectrum displayed a pseudomolecular ion peak at 543 $[M-H]^-$, suggesting a molecular formula $C_{27}H_{28}O_{12}$. All of the NMR spectral data indicated that compound **174** was a 4,5-dicaffeoylquinic acid ethyl ester. The presence of an EtO group was supported by the appearance of q-like signal at δ ca. 4.16 integrating for two protons and a triplet signal at δ 1.26 integrating for three protons. By comparison of the chemical shifts of the EtO group with literature data reported, it was concluded that it was part of the carboxylic unit. Thus, compound **174** was identified as ethyl 4,5-dicaffeoylquinate. The NMR spectral data of the isolated compound in the present study match well with those reported earlier.[191]

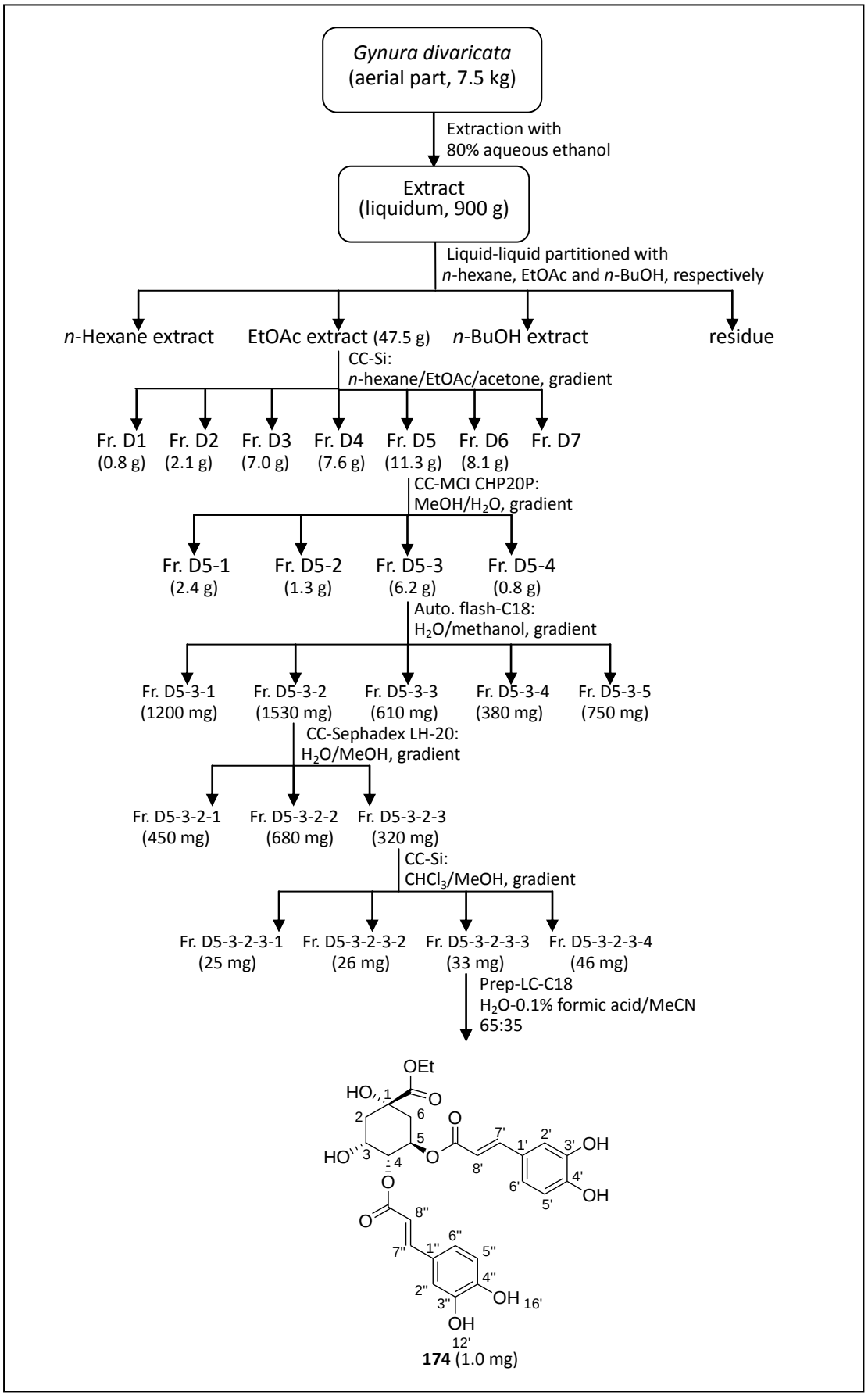
The comparison of characteristic NMR data of compound **174** with literature data is listed in the Table 3.49.[191]

Table 3.49 Comparison of characteristic ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of ethyl 4,5-dicaffeoylquinate **174** with literature data (200/50 MHz, acetone-d_6)[191]

Position	This work		Literature data	
	^{1}H	^{13}C	^{1}H	^{13}C
1		75.6		75.86
2	2.03-2.36 (2H, m)	38.3	2.06 (2H, m)	38.18
3	4.34-4.36 (1H, m)	68.5	4.40 (1H, d, *J* = 3.0 Hz)	68.23
4	5.09-5.13 (1H, m)	69.1	5.11 (1H, dd, *J* = 9.3, 3.0 Hz)	68.86
5	5.50-5.57 (1H, m)	74.8	5.66 (1H, ddd, *J* = 9.3, 9.3, 3.9 Hz)	75.29
6	2.03-2.36 (2H, m)	38.4	2.06(2H, m)	39.28
COO$\underline{CH_2}$	4.13-4.21 (2H, m)	62.7	4.17 (2H, q, *J* = 7.0 Hz)	61.88
$CH_2\underline{CH_3}$	1.26 (3H, t, *J* = 7.2 Hz)	14.3	1.26 (3H, t, *J* = 7.0 Hz)	14.32
$\underline{C}OO$		174.7		173.66
1’/1”		127.6/127.5		127.46
2’/2”	7.04/7.01 (2H, d, *J* = 1.7 Hz)	116.5	7.14/7.13 (2H, d, *J* = 1.9 Hz)	115.34/115.14
3’/3”		146.8		145.30
4’/4”		149.8/149.7		148.84
5’/5”	6.76 (2H, d, *J* = 8.3 Hz)	115.1	6.88/6.84 (2H, d, *J* = 7.9 Hz)	116.32
6’/6”	6.90-6.95 (2H, m)	123.1	7.02/7.00 (2H, d, *J* = 1.9, 7.9 Hz)	122.64
7’/7”	7.61/7.51 (2H, d, *J* = 16.0 Hz)	147.7/147.6	7.58/7.54 (2H, d, *J* = 15.8 Hz)	146.28
8’/8”	6.30/6.18 (2H, d, *J* = 16.0 Hz)	114.6/114.5	6.27/6.24 (2H, d, *J* = 15.7 Hz)	114.00/113.40
9’/9”		168. 5/167.9		166.96/166.63

Scheme 3.8 describes the complete isolation of compound **174** from the aerial parts of *G. divaricata*.

Scheme 3.8 The complete isolation of compound **174** from *G. divaricata*



174 (1.0 mg)

Chapter 4

INVESTIGATION OF THE VOLATILE CONSTITUENTS OF DIFFERENT *GYNURA* SPECIES

4.1 Introduction

The genus *Gynura* belongs to the family Asteraceae, comprising approximately 40 species mainly distributed in Asia, Africa and Australia, of which 10 species were recorded in the south of China.[196] Besides those species, *Gynura medica* is a newly found plant named by Yang and Wu.[197] Many *Gynura* species are edible plants native to Asia, aerial parts are consumed in traditional dishes.[153,198] Besides, some of them have been used heavily as traditional Chinese medicines in the treatment of hemostasis,[199.200] hypertension,[201] and diabetes mellitus.[174] In our investigation of natural medicines used in the traditional Chinese medical system for treatment of diabetes,[52,151] a tea made from the fresh leaves of *G. bicolor* and/or *G. divaricata* was found to have excellent anti-hyperglycemia effects. *G. bicolor* also produces a pleasant flavor, associated with slightly green and earthy aromas.[154]

To our knowledge, three studies investigated the volatile constituents from *Gynura* species, i.e. *G. cusimbua*[202] and *G. bicolor*.[153,154] Shimizu analyzed and compared the volatiles from extracts of leaves of field-grown plants and shoot cultures of *G. bicolor* using solvent-assisted flavour evaporation (SAFE) coupled with gas chromatography-mass spectrometry (GC-MS) identification.[154]

Essential oils are volatile secondary metabolites produced by aromatic plants and play an important role in the protection and life cycle of plants.[203] Due to their interesting biological effects, they play an important role in traditional pharmacopoeia. The chemical composition of essential oils is determined by local environmental factors influencing plant growth.[203] The extraction techniques of essential oils are variable and under continuous development and can have an influence on the chemical composition which is typically determined by GC-MS analyses. As a solvent-free sampling technique for the determination of volatiles in plant essential oils, solid phase microextraction (SPME) has several advantages, compared with other extraction techniques such as traditional steam distillation-solvent extraction, such as high sensitivity and a small sample volume for rapid analysis of volatiles.[204,205,206] When volatile profiles resulting from SPME techniques are compared with profiles obtained in essential oil research through conventional distillation techniques, it has to be taken into account that the profile acquired using an SPME fiber is influenced by several extraction parameters such as the type of fiber (different partition coefficients), time and temperature.[206] Nevertheless, the advantage of the need of a relative small sample volume makes SPME more useful facing plants with limited availability, specifically the wild types. Therefore, for the first time, the volatiles from fresh leaves of *Gynura divaricata* and *G. bicolor* were studied using a SPME method coupled with GC-MS. The present work reports the volatile compounds of *G. divaricata, G. bicolor* collected from two different origins, Nanjing and Nanping in the east of China. An analysis of the volatiles from leaves of *Gynura medica* was also performed. These three plants have all been reported as having hypoglycemic potential for the treatment of diabetes in a bioassay test.[151,207,208]

4.2 Experimental

4.2.1 Plant material

Gynura species from Nanping area were collected in September 2011 from the countryside of Jianou county, in the southeast of the Wuyi Mountain, Nanping, China. Species from Nanjing area were collected in October 2011 in Nanjing Botanical Garden Mem. Sun Yat-sen, in the south of the Zijin Mountain, Nanjing, China. The plants were identified by Professor Guo Rong-lin at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. The voucher specimens of three *Gynura* species (No. 510826-1~5) were deposited in the herbarium, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences.

Fresh leaves of *Gynura* species were air dried and then ground to a powder, weighted and subjected to headspace sampling.

4.2.2 Headspace sampling

The volatile metabolites from 2.0 g of dry leaf powder of the plant were extracted by headspace SPME during 30 min at 40 °C with a 50/30 µm DVB/Car/PDMS fiber (Supelco, Bornem, Belgium) and desorbed for 2 min at 250 °C. SPME extraction and desorption were performed automatically by means of a Multipurpose Sampler (MPS-2, Gerstel).[205] The sample extractions were conducted in triplicate.

4.2.3 Analysis of the headspace extracts by GC-MS

GC-MS analyses of the SPME extracts were performed with an Agilent 6890 GC Plus coupled to a quadrupole mass spectrometer 5973 MSD (Agilent Technologies, Diegem, Belgium), and equipped with a DB-5 capillary column (30 m length × 0.25 mm id; 0.25 µm film thickness). Working conditions were: injector temperature 250 °C, transfer line to MSD 260 °C, carrier gas (He) 1 mL min^{-1}; SPME desorption in a CIS-4 PTV injector (Gerstel) in split mode (10 : 1); ionisation: EI 70 eV; acquisition parameters: scanned m/z: 40-200 (2-15 min), 40-300 (15-20 min), 40-350 (> 20 min); oven temperature starts at 60 °C, programmed from 60 °C to 160 °C at 3 °C min^{-1}, from 160 °C to 250 °C at 10 °C min^{-1}, hold 2 min. Retention indices were calculated using co-chromatographed standard hydrocarbons. The individual compounds were identified by comparing their retention indices, relative to C6-C18 n-alkanes, with literature values and by comparing their mass spectra and retention times with those of authentic samples or with data already available in the NIST 05 library and the literature.

4.3 Results and Discussion

Via headspace SPME/GC-MS analysis, a total of 55 different components were identified from fresh leaves of different *Gynura* species which were air dried. The constituents were identified by comparison of the mass spectrum with those of the computer mass library NIST 05 and/or calculated retention indexes (RI) with those reported in the literature (listed in Table 4.1). For *G. bicolor*, 47 and 33 compounds were identified from the species originating from Nanping and Nanjing, respectively. The main volatile

constituents of *G. bicolor* from Nanping were β-caryophyllene (203×10^6 peak area), α-caryophyllene (42×10^6), α-copaene (34×10^6), carvone (31×10^6) and limonene (31×10^6) as monoterpenes or sesquiterpenes. β-Caryophyllene (175×10^6), α-pinene (72×10^6), α-copaene (52×10^6), α-caryophyllene (50×10^6) and β-pinene (10×10^6) were the main monoterpenes or sesquiterpenes present in *G. bicolor* from Nanjing.

The detected major volatiles in *G. divaricata* from Nanjing were qualitatively the same as for *G. bicolor* from Nanjing, but in smaller amounts. The volatile profiles of *G. divaricata* and *G. bicolor* from Nanping were more diverse. Instead of carvone and α-caryophyllene for *G. bicolor*, o-cymene was detected in *G. divaricata*, besides β-caryophyllene, limonene and α-copaene.

In addition, for the first time, the volatiles of the fresh, air dried, leaves of *G. medica* collected from Nanjing were also identified via SPME/GC-MS, with γ-cadinene (81×10^6), β-caryophyllene (39×10^6), elixene (21×10^6) and monoterpene limonene (20×10^6) as the major components. The volatile profile of this third *Gynura* species is clearly different from that of *G. bicolor* and *G. divaricata* from the same origin. Overall, these results indicate that the volatile profile of *Gynura* species is not only species related, but is also influenced by the local environmental growth conditions. Since many studies proved the hypoglycemic effects of volatiles from plants,[211,212] the further biotesting of total volatile extracts from *Gynura* species for antidiabetic activity will be of significant interest.

Table 4.1 Composition of the volatile compounds (GC-MS peak area ×10^6) from fresh (air dried) leaves of *Gynura* species identified by SPME/GC-MS.

Compound[a]	RI_{exp}[b]	RI_{lit}[c]	ID[d]	*G. bicolor* (Nanjing)	*G. bicolor* (Nanping)	*G. divaricata* (Nanjing)	*G. divaricata* (Nanping)	*G. medica* (Nanjing)
Toluene[e]	766		MS		4.3		5.3	3.1
Hexanal	801	801	MS, RI	1.7	3.0	0.8	2.0	1.6
(*E*)-2-Hexenal	847	846	MS, RI	0.3	0.6	0.3	0.7	4.9
(*E*)-2-Hexenol	857	854	MS, RI		0.1		0.2	2.5
1-Hexanol	861	863	MS, RI	0.2	0.4	0.2	0.8	2.1
2,6-Dimethylpyridine[e]	877		MS		0.4		0.8	
2-Butylfuran[e]	889		MS	0.3	0.1	0.2	0.3	0.3
Styrene[e]	893		MS		0.2		0.3	0.2
3-Nonene[e]	896		MS		5.7		8.7	6.9
Heptanal	901	901	MS, RI	0.2	0.2	0.2	0.3	0.1
(*E*,*E*)-2,4-Hexadienal	909	907	MS, RI	0.2	0.3	0.3	0.6	0.8
α-Thujene	924	924	MS, RI	0.3	0.3	0.3	0.6	0.5
α-Pinene	932	932	MS, RI	72.4	1.8	49.7	1.6	7.7
(*Z*)-2-Heptenal[e]	952		MS		0.8		0.6	
Benzaldehyde	959	952	MS, RI	1.1	0.5	0.7	0.5	0.6
Sabinene	970	969	MS, RI	0.6	0.5	0.3	0.7	0.7
β-Pinene	977	974	MS, RI	9.8	0.6	13.8	1.0	1.9
Sulcatone	980	985	MS, RI	2.5	0.4	2.8	0.6	0.6
Myrcene	987	988	MS, RI	3.9	4.0	1.8	4.2	4.9
Ethyl caproate	996	996	MS, RI		0.6		1.0	1.2
4-Carene[209]	999	1001	MS		0.5		0.8	0.8
Octanal	1002	1001	MS, RI		0.2		0.3	0.3
α-Phellandrene	1006	1002	MS, RI		2.8		3.9	4.4

δ-3-Carene	1008	1008	MS, RI	0.2		1.7		
α-Terpinene	1016	1014	MS, RI		3.5		4.8	6.1
ortho-Cymene	1023	1022	MS, RI	0.5	9.0	1.0	10.9	12.9
Limonene	1027	1024	MS, RI	0.5	30.8	0.4	21.0	20.1
β-Phellandrene	1029	1025	MS, RI		2.6		5.1	4.0
γ-Terpinene	1055	1054	MS, RI		3.1		3.8	4.8
1-Undecene[210]	1089	1092	MS	5.2	0.5	0.2	0.5	1.0
Linalool	1097	1095	MS, RI					0.3
Nonanal	1102	1100	MS, RI		1.3		0.8	0.6
Citronellal	1148	1148	MS, RI					0.6
Menthone	1153	1148	MS, RI		3.1		1.4	0.8
Menthol	1174	1167	MS, RI		1.3		1.6	1.0
Methyl salicylate	1187	1190	MS, RI					0.5
Carvone	1239	1239	MS, RI		30.9		4.5	1.3
3-Cyclohexen-1-one, 2-isopropyl-5-methyl-[e]	1249		MS	1.6		1.8		0.3
α-Cubebene	1341	1345	MS, RI	0.9		0.4		2.5
Cyclosativene	1363	1369	MS, RI	4.4	2.0	1.8	1.4	1.5
α-Copaene	1370	1374	MS, RI	51.6	33.5	23.3	17.6	12.9
β-Elemene	1383	1389	MS, RI	1.5	1.9	0.3	0.2	4.0
α-Gurjunene	1400	1409	MS, RI			2.0	2.2	
β-Caryophyllene	1413	1417	MS, RI	174.9	203.4	43.7	48.2	38.7
α-Caryophyllene	1449	1454	MS, RI	49.5	41.7	9.0	5.0	17.2
Unknown	1468			4.6	4.5	1.7	1.4	0.7
γ-Muurolene	1474	1478	MS, RI	2.1	3.9	0.6	0.6	6.6
Germacrene D	1476	1484	MS, RI		0.4		0.6	11.4
β-Selinene	1482	1489	MS, RI	1.6	0.6	1.2	1.1	0.9

Elixene[e]	1488		MS	9.6	6.7	2.7	0.8	21.1
α-Muurolene	1492	1500	MS, RI	1.3	1.1	1.3	1.4	0.4
α-Farnesene	1499	1505	MS, RI		0.1			0.5
γ-Cadinene	1512	1513	MS, RI	9.1	9.6	5.6	4.3	80.8
Dihydroactinidiolide[e]	1518		MS	0.5	0.6	0.3	0.5	
Spathulenol	1569	1577	MS, RI	6.4	7.7	1.7	1.2	6.4
Caryophyllene oxide	1574	1582	MS, RI	8.7	16.3	2.2	4.6	0.3
Unknown	1626							16.1

[a]Compounds are listed in order of their retention time on a DB-5 column.

[b]RI_{exp} = retention indices are determined using n-alkanes (C6-C18) as reference materials.

[c]RI_{lit} = relative retention indices taken from Adams and/or Nist 05 for DB-5 capillary column.

[d]Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Adams and NIST 05; RI, by comparison of calculated RI with those reported in the literature.

[e] Tentative identification

Chapter 5

SCREENING OF THE BIOLOGICAL ACTIVITY OF THE NATURAL PRODUCTS ISOLATED FROM *GYNURA BICOLOR* AND *GYNURA DIVARICATA*

5.1 Introduction

One of the therapeutic approaches to correct the increased blood glucose level in type 2 diabetes mellitus is the management of postprandial hyperglycemia by reducing the production and absorption of glucose by inhibition of carbohydrate-digestion enzymes, such as α-glucosidase, maltase and sucrase.[213] The close link between α-glucosidase and diabetes has led to a large number of studies dedicated to search for these enzymic inhibitors. Several natural α-glucosidase inhibitors including acarbose, voglibose and miglitol are clinically used.[214]

Protein tyrosine phosphatase-1B (PTP1B) is a widely distributed, nontransmembrane protein tyrosine phosphatase, originally identified in human placenta.[215] It was proven to be negative regulator of insulin receptor signaling, which has emerged as a potential therapeutic target for treatment of type 2 diabetes and obesity.[216] Therefore, the development of such phosphatase inhibitors will be beneficial for drug discovery for the treatment of type 2 diabetes.

As a folk medicine, *Gynura bicolor* and *Gynura divaricata* have been used to prevent and treat diabetes for decades in China. One research was investigated to evaluate different extracts of *Gynura divaricata* on inhibition of α-amylase and α-glycosidase involved in the pathogenesis of hyperglycemia. The results suggest that *G. divaricata* may have potential hypoglycemic applications.[207] However, the studies on antidiabetic effects of *Gynura* species were mainly focused on the activity of the extract and the active components of the extract were not ascertained.[217,218,219] In addition, cinnamic acid and caffeic acid **76**, the precursors of all caffeoyl derivatives,[220] together with 5-caffeoylquinic acid (chlorogenic acid, **74**)[221] which were found to be present in the studied *Gynura divaricata*, have been well studied for PTP1B inhibitory activities. With the combined use of structure and medicinal chemistry, it was decided to study all the natural products isolated from *Gynura bicolor* and *Gynura divaricata* by enzyme assay-biotesting, to obtain small-molecule α-glucosidase and PTP1B inhibitors with the requisite potency and selectivity.

5.2 Screening of the natural products as α-glucosidase inhibitors

The inhibitory activity against α-glucosidase (Type I, Sigma-Aldrich) was measured with a slight modification of the reported method.[222] To each well of a 96-well microtiter plate was added 10 μl of α-glucosidase [1 U/ml in phosphate buffer (PBS), pH 6.8], 28 μl of PBS, 2 μl of the sample solution in 4% MeOH. The mixture was incubated at 37 °C for 10 min, then 4-nitrophenyl α-D-glucopyranoside (10 μl, 10 mM in PBS) was added, and the mixture was incubated for additional 35 min. The absorbance (A) of each well was measured at 405 nm with a microplate spectrophotometer (Infinite F50, Tecan). The inhibition activity was calculated using the following formula: Inhibition (%) = $[1-(A_{sample}/A_{control})] \times 100\%$. The positive control was acarbose (Bayer) which was found to have an IC_{50} value of 1.38 mM against the same enzyme.

5.3 Screening of the natural products as Protein Tyrosine Phosphatase 1B inhibitors

All compounds were dissolved in MeOH at a concentration of 10 mg/mL and 2 μl was added to each well. The final concentration of solvent did not influence the activity of PTP1B. The assay was performed in a 96-well clear polystyrene microplate (Corning) according to a published procedure with slight modifications.[223] Each well contained 0.05 μg PTP1B (Enzo Life Sciences, Inc.), 1.5 mM Insulin Receptor ß residues (IR5, Enzo Life Sciences, Inc.) and 100 mM MES buffer [pH 6.0, 300 mM NaCl, 2 mM N,N,N',N'-ethylenediaminetetraacetate (EDTA), 2 mM dithiothretiol (DTT), 0.1% NP-40). The final volume of the mixture was 100 μL. The reaction was initiated by addition of IR5, incubated at 37 °C for 30 min, and terminated with the addition of concentrated phosphate detection reagent (BIOMOL RED, Enzo Life Sciences, Inc.). The absorbance (A) of each well was measured at 620 nm with a microplate spectrophotometer (Infinite F50, Tecan). The inhibition activity was calculated using the following formula: Inhibition (%) = $[1-(A_{sample}/A_{control})] \times 100\%$. The assays were performed in triplicate for all samples. A PTP1B inhibitor, SURAMIN (Enzo Life Sciences, Inc.) was used as the positive control.

5.4 Results and discussion

5.4.1 Inhibitory activities of isolated compounds against α-glucosidase

Among the isolated compounds, 3,4-dicaffeoylquinic acid **167**, 4,5-dicaffeoylquinic acid **73**, methyl 3,4-dicaffeoylquinate **168** and methyl 4,5-dicaffeoylquinate **170** exhibited significant inhibitory activities against α-glucosidase (Table 5.1). Their IC_{50} values were much lower than that of acarbose, in particular, while methyl 3,4-dicaffeoylquinate **168** and methyl 4,5-dicaffeoylquinate **170** showed IC_{50} values of 12.23 ± 0.64 μM and 13.08 ± 0.86 μM, respectively. It should be noted that methyl 3,4-dicaffeoylquinate **168** and methyl 4,5-dicaffeoylquinate **170** are derivatives of 3,4-dicaffeoylquinic acid **167** and 4,5-dicaffeoylquinic acid **73**, respectively. From this result, it was clear that their activities concerning inhibition of α-glucosidase increased almost ten times after esterification. Besides, the flavonol quercetin **35** also showed significant activity against α-glucosidase.

Table 5.1 Inhibitory activities of isolated compounds against α-glucosidase

Compounds	IC_{50} values
3,4-Dicaffeoylquinic acid **167**	187.2 ± 12.9 μM
4,5-Dicaffeoylquinic acid **73**	130.8 ± 10.3 μM
Methyl 3,4-dicaffeoylquinate **168**	12.23 ± 0.64 μM
Methyl 4,5-dicaffeoylquinate **170**	13.08 ± 0.86 μM
Quercetin **35**	36.09 ± 2.81 μM
Acarbose	1.38 mM

Notes: Data of other isolated compounds showing no activity on inhibition of α-glucosidase were not listed in this table. IC_{50} is the concentration required to produce 50% inhibition of the enzyme activity. Values represent the means ± standard deviation (SD) of n = 3 duplicate assays.

5.4.2 Inhibitory activities of isolated compounds against PTP1B

As shown in Table 5.2, seven of forty-one isolated compounds from *G. bicolor* and *G. divaricata* exhibited an inhibition effect on PTP1B. 3,5-Dicaffeoylquinic acid **72** and 4,5-dicaffeoylquinic acid **73** showed a considerable inhibitory effect on PTP1B, compared with the positive control of SURAMIN against the same enzyme in a parallel assay. On the contrary, 3,4-dicaffeoylquinic acid **167**, the isomer of the above dicaffeoylquinic acids, displayed no inhibition activity against PTP1B. In addition, only methyl 3,4-dicaffeoylquinate **168** showed very limited inhibition activity on PTP1B, suggesting that esterification will decrease their abilities on inhibition of PTP1B. *p*-Coumaric acid **165**, and its derivative 5-O-*p*-coumaroylquinic acid **171** showed comparable activities on the inhibition of PTP1B, which suggested that the quinic acid part did not influence the effect. Total DCQ, the total dicaffeoylquinic acid derivatives extract (Fr. D5-3-2-2) from *G. divaricata*, also showed comparable inhibition effect, while 3,5-dicaffeoylquinic acid **72** and 4,5-dicaffeoylquinic acid **73** in this extract were deduced to be responsible for the inhibition efficacy. Among the other active compounds, megastigmane-type norisoprenoid compound vomifoliol **153** and the flavonoid compound quercetin 3-O-β-D-glucoside **36** exhibited the highest inhibition against PTP1B, suggesting the particular molecular interaction between the compounds and the enzyme.

Table 5.2 Inhibitory activities of isolated compounds against PTP1B

Compounds	PTP1B inhibition rate
Vomifoliol **153**	68.90 %
5-O-*p*-Coumaroylquinic acid **171**	27.30 %
3,5-Dicaffeoylquinic acid **72**	41.60 %
4,5-Dicaffeoylquinic acid **73**	58.20 %
Methyl 3,4-dicaffeoylquinate **168**	3.70 %
p-Coumaric acid **165**	28.80 %
Quercetin 3-O-β-D-glucoside **36**	68.10 %
Total DCQ	57.30 %
SURAMIN	44.60 %

Notes: Sample "Total DCQ" is the total dicaffeoylquinic acid derivatives extract (Fr. D5-3-2-2) from *G. divaricata.* Data of other isolated compounds showing no activity on inhibition of PTP1B were not listed in this table.

Based on the above, the hydroxycinnamic acid conjugates isolated from the aerial part of *G. divaricata* seem more active for the inhibition of the studied enzymes. The influence of the methyl ester, the linkage position between the quinic acid and the cinnamoyl group, and the number of caffeic acid moieties in the structure, was evaluated. By comparison of the reported activities of quinic acid, caffeic acid and coumaric acid derivatives,[224,225] it was suggested that the number of caffeoyl groups attached to a quinic acid core were important for the α-glucosidase inhibitory potency. Besides, the methyl esters of the isolates always produce more significant effect on the inhibition of α-glucosidase. Regarding to the natural phenolics on the inhibition of PTP1B, flavonoids and caffeic acid were proven to show strong inhibitory activity.[226] The result from the

present study would be a complementary evidence for the use of *G. species* for antidiabetes.

Because of the small amounts of megastigmane-type norisoprenoids isolated from *G. bicolor*, the research work on their biological activity could not be studied further. However, it is interesting to note that (6*S*,9*R*)-roseoside, which is an isomer of (6*S*,9*S*)-roseoside (**155**) currently isolated, had previously been shown to increase insulin secretion from INS-1 β-cells.[227]

The biological screening suggested that the hydroxycinnamic acid conjugates in *G. divaricata* were the major constituents responsible for its hypoglycemic activity. Meanwhile, with regard to our previous pharmacological investigation of *Gynura* species on alloxan-diabetic mice,[151] it was deduced that its action was possibly related to the protection of pancreatic β-cells or stimulation of undamaged cells to secrete more insulin. Recent studies have indicated that many plant secondary metabolites, such as hydroxycinnamic acid conjugates, have radical-scavenging activity, which is beneficial for alleviation of the high oxidative stress in cytotoxic-induced diabetic animal models.[184,186] To further investigate the potential antioxidant capacity of chemical constituents from *G. bicolor* and *G. divaricata*, the biotesting of isolated natural products on reactive oxygen species such as the hydroxyl radical (·OH), the superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) in vitro is still going on. The results of the biological activity testing will be communicated later.

Chapter 6

SUMMARY

Historically, natural products provide considerable value to the primary health care of a large part of the world's population. Chemical research directed towards the isolation of active constituents from plants used in traditional medicine continues to be a very important source of potentially useful therapeutic agents.

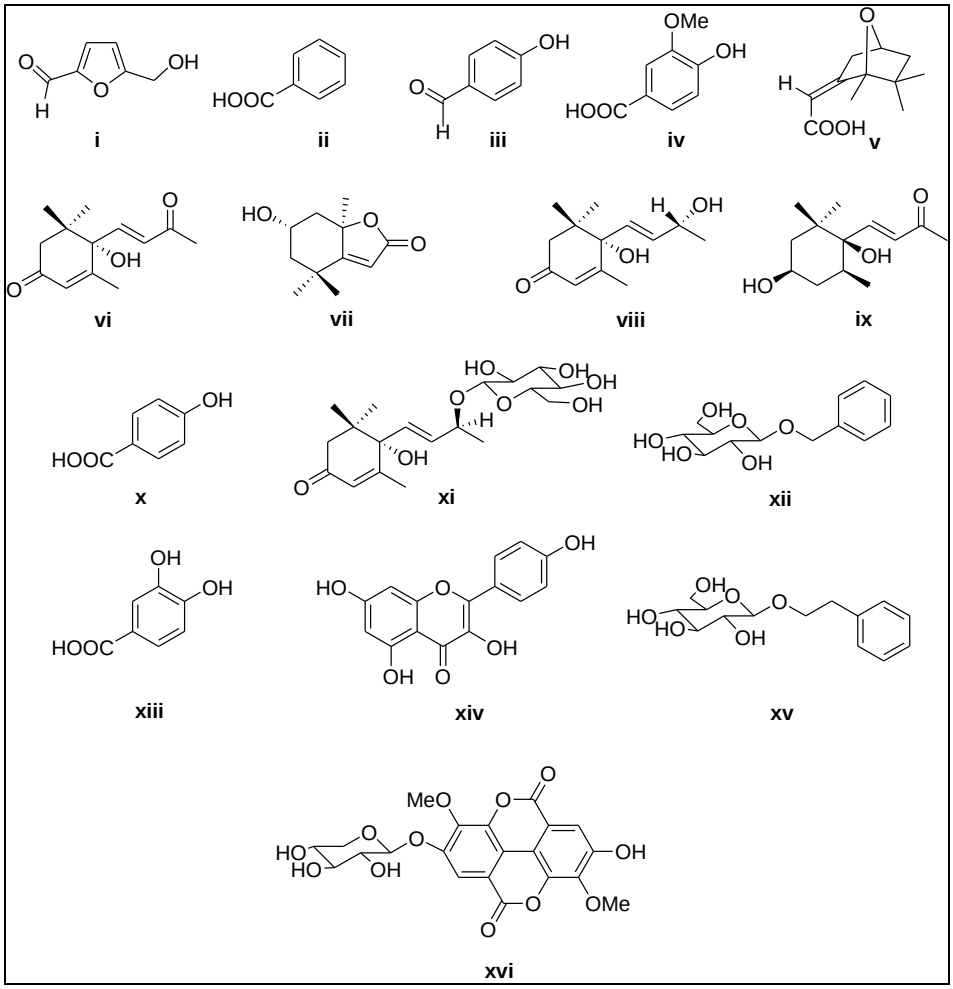
The rapidly increasing incidence of diabetes mellitus is becoming a serious threat to human health in all parts of the world. There has been a growing interest in hypoglycemic agents from natural products, and their activities on diabetic related complications and their mechanism of action attract more attention as well. To gain insight in the antidiabetes activities of natural products and their multiple mechanisms of action, an overview of selected natural flavonoids with validated hypoglycemic properties and their use in the treatment of diabetic complications was presented in Chapter 1. The knowledge offered should help to provide leads to the ultimate goal of developing new, more efficient therapeutic drugs for the treatment of diabetes mellitus and its complications.

Several plant species from the genus *Gynura* are used as folk medicines in China. Among them, *Gynura bicolor* and *Gynura divaricata* both displayed an excellent antidiabetic effect. As local secret recipes, *G. bicolor* and *G. divaricata* have been used to treat diabetes in Jiangsu, Zhejiang and Sichuan province in south China for decades. Our research group in China performed pharmacological tests to prove the antihyperglycemic effect of these two plants. These tests revealed that both ethyl acetate and butanol extracts of *G. bicolor* showed a significant effect on lowering the blood glucose level in normal and alloxan-diabetic mice, while the ethyl acetate and butanol extracts of the aerial part of *G. divaricata* were effective on lowering the blood glucose level in alloxan-diabetic mice in a low dosage. However, the phytochemical study of these species is scarcely reported in literature. Therefore, this PhD-thesis was designed to perform further research on the isolation of the active chemical constituents of these two species.

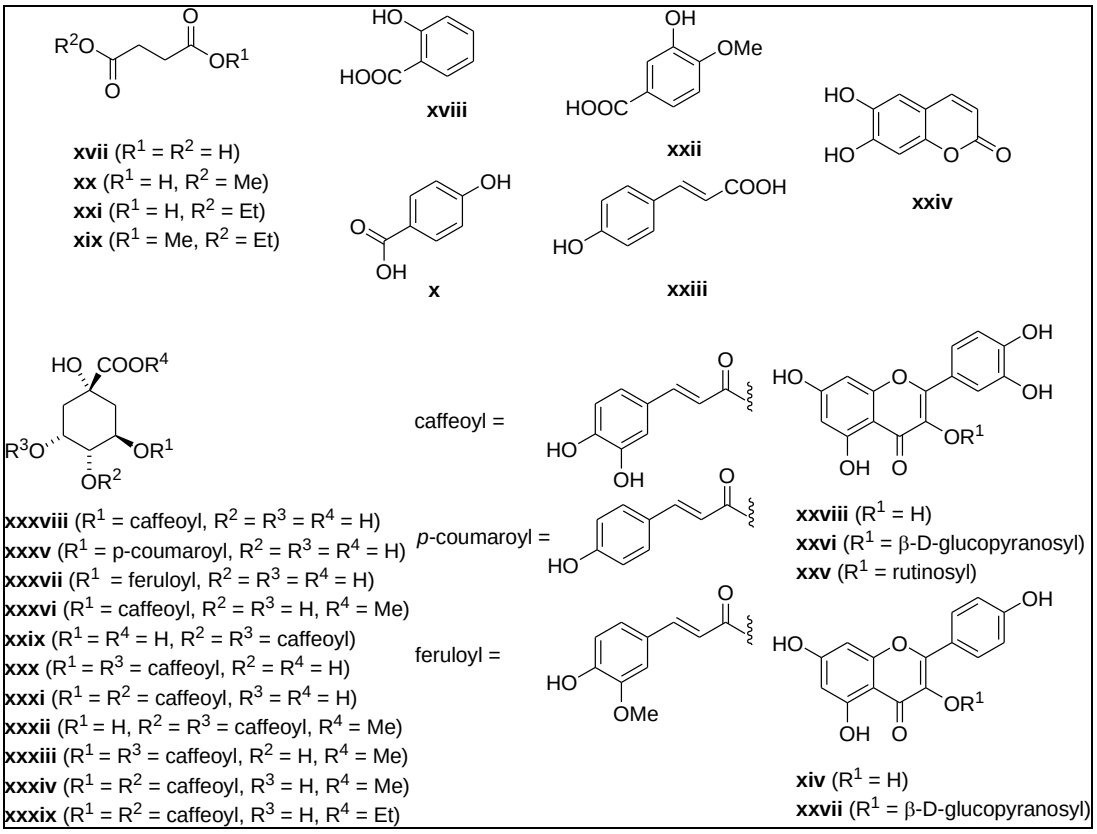
This work describes the isolation and structure elucidation of natural products from *G. bicolor* (Chapter 2) and *G. divaricata* (Chapter 3). Due to the fact that *G. bicolor* also produces a pleasant flavor, three different *Gynura* species from two Chinese origins were collected and their volatile constituents were investigated using a SPME method coupled with GC-MS (Chapter 4). In addition, all the isolated natural products were subjected to a bioactivity screening for antidiabetes activity (α-glucosidase and PTP1B inhibition), which was analyzed in Chapter 5.

The extraction, fractionation, isolation and final purification of the natural products were achieved by means of liquid-liquid partitioning, column chromatography with different packing materials (silica gel, MCI CHP20P gel, Sephadex gel, C18), automatic flash and preparative high pressure liquid chromatography (prep-HPLC). ^{1}H NMR, ^{13}C NMR spectrometry (including ^{1}H-^{1}H COSY, HSQC, HMBC, DEPT, etc) and mass spectrometry were used to elucidate the structure of the isolated natural products.

Chapter 2 describes the phytochemical investigation of *G. bicolor*. The fractionation and isolation of the CH_2Cl_2 and EtOAc extracts of the aerial parts of *G. bicolor* resulted in sixteen pure compounds. Their structures were determined spectroscopically as 5-(hydroxymethyl)furfural **i**, benzoic acid **ii**, 4-hydroxybenzaldehyde **iii**, vanillic acid **iv**, ficusic acid **v**, dehydrovomifoliol **vi**, loliolide **vii**, vomifoliol **viii**, boscialin **ix**, 4-hydroxybenzoic acid **x**, roseoside **xi**, benzyl β-D-glucopyranoside **xii**, protocatechuic acid **xiii**, kaempferol **xiv**, 2-phenylethyl β-D-glucopyranoside **xv** and 3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside **xvi**. It should be noted that all these compounds, except 4-hydroxybenzoic acid **x** and kaempferol **xiv** were isolated for the first time from this plant, and no evidence could be found of the previous reported presence of megastigmane-type norisoprenoids in the genus *Gynura*.



The phytochemical investigation of natural products of *G. divaricata* (Chapter 3) led to the isolation of twenty-five compounds. They were characterized by spectrometric methods as one organic acid (succinic acid **xvii**) and three of its derivatives (ethyl methyl succinate **xix,** methyl succinate **xx** and ethyl succinate **xxi**), four phenolic acids (salicylic acid **xviii**, isovanillic acid **xxii,** 4-hydroxybenzoic acid **x** and *p*-coumaric acid **xxiii**), one coumarin (esculetin **xxiv**), five flavonoids (quercetin **xxviii**, kaempferol **xiv**, rutin **xxv**, quercetin 3-O-β-D-glucoside **xxvi** and kaempferol 3-O-β-D-glucoside **xxvii**) and eleven quinic acid derivatives (5-O-caffeoylquinic acid **xxxviii**, methyl 5-O-caffeoylquinate **xxxvi**, 5-O-feruloylquinic acid **xxxvii** and 5-O-*p*-coumaroylquinic acid **xxxv,** 3,4-dicaffeoylquinic acid **xxix**, 3,5-dicaffeoylquinic acid **xxx**, 4,5-dicaffeoylquinic acid **xxxi**, methyl 3,4-dicaffeoylquinate **xxxii**, methyl 3,5-dicaffeoylquinate **xxxiii**, methyl 4,5-dicaffeoylquinate **xxxiv** and ethyl 4,5-dicaffeoylquinate **xxxix**). It should be noted that compounds **x**, **xvii-xxiv**, **xxvi**, **xxix** and **xxxi-xxxix** were isolated for the first time from this plant.



In order to study the active natural products in these two *Gynura* species responsible for their antidiabetes properties, the individual compounds were tested for the inhibition of α-glucosidase and Protein Tyrosine Phosphatase 1B (PTP1B). Among the isolated compounds, 3,4-dicaffeoylquinic acid **xxix**, 4,5-dicaffeoylquinic acid **xxxi**, methyl 3,4-dicaffeoylquinate **xxxii** and methyl 4,5-dicaffeoylquinate **xxxiv** exhibited significant inhibitory activities against α-glucosidase, with IC_{50} values of 187.2 ± 12.9 μM, 130.8 ± 10.3 μM, 12.23 ± 0.64 μM and 13.08 ± 0.86 μM, respectively. Besides, the flavonol quercetin **xxviii** also showed significant activity against α-glucosidase. Regarding the inhibition activity of isolated compounds against PTP1B, 3,5-dicaffeoylquinic acid **xxx** and 4,5-dicaffeoylquinic acid **xxxi** showed considerable inhibitory effect on PTP1B. *p*-Coumaric acid **xxiii** and 5-O-*p*-coumaroylquinic acid **xxxv** also showed inhibition effects against PTP1B. Based on these findings, the hydroxycinnamic acid conjugates were deduced to be potentially responsible for their antidiabetes activity of *Gynura divaricata*. However, megastigmane-type norisoprenoid compounds from *G. bicolor* showed a slight activity against the above two enzymes, except for vomifoliol **viii** which exhibited a high inhibition of PTP1B, suggesting a particular molecular interaction between the compounds and the enzyme.

Another part of the research work was focused on the volatile compounds of *G. divaricata* and *G. bicolor* collected from two different origins, Nanjing and Nanping in the east of China. Analysis of the volatiles from leaves of *Gynura medica* was also performed. Via headspace SPME/GC-MS analysis, a total of 55 different components were identified from fresh leaves of different *Gynura* species. By qualitative and quantitative analysis, it was indicated that the volatile profile of *Gynura* species is not only species related, but is also influenced by the local environmental growth conditions.

The future perspectives of further research should be focused on the completion of the phytochemical work on the *Gynura* species. Preliminary in depth LC-MS profiling indicates that several minor isomers (both structural and stereo chemicals) are available of the hydroxycinnamic acid conjugates. Each isomer of the hydroxycinnamic acid conjugates needs to be structurally confirmed, which contributes to complete and comprehensive elucidation of the secondary metabolites from the *Gynura* plants and their potential for antidiabetic effect, due to their high concentrations. Furthermore, the bioactivity of the hydroxycinnamic acid conjugates needs to be further investigated for evaluation of the full ethnomedicinal potential of *Gynura* species.

In recent years, a serious concern regarding *Gynura* plants (Asteraceae), especially *G. japonica*, exists due to the presence of hepatotoxic pyrrolizidine alkaloids (HPAs), which can bind to DNA or proteins after being activated in the liver.[228] In 1989, the World Health Organization issued a Health and Safety Guide on utilization of HPAs-containing herbs to prevent the risk of human exposure to these alkaloids, and these herbs are strictly limited in western developed countries.[229] Previous phytochemical investigation of *G. divaricata* showed the presence of integerrimine and usaramine classified as

retronecine type HPAs,[22] indicating the toxic risk for using this medicinal plant. Therefore, a full investigation focusing on profiling of the HPAs in *Gynura* species and the safety assessment of the *Gynura* plants is necessary in future work.

Natural products will continue to play a significant role as lead compounds for drug discovery, combined with new technologies such as combinatorial (bio)synthesis and gene mining. A bright future for natural products in drug development for treatment of all human being's diseases, including diabetes, is foreseen.

Chapter 7

SAMENVATTING

Gynura bicolor en *G. divaricata* zijn, als lokale geheime recepten, in het zuiden van China gedurende decennia gebruikt om diabetes te behandelen. Om de actieve natuurproducten verantwoordelijk voor de antidiabetes eigenschappen te achterhalen, werd dit doctoraatsonderzoek uitgevoerd betreffende de isolatie van de actieve chemische bestanddelen van deze twee soorten *Gynura*. Een ander deel van het onderzoek was gericht op de vluchtige verbindingen van *G. bicolor* en *G. divaricata* verzameld uit twee verschillende oorsprongen, Nanjing en Nanping in het oosten van China.

De fractionering en isolatie van de CH_2Cl_2 en EtOAc extracten van de bovengrondse delen van *G. bicolor* resulteerde in zestien zuivere verbindingen. Hun structuren werden spectroscopisch bepaald als: 5-(hydroxymethyl)furfural **i**, benzoëzuur **ii**, 4-hydroxybenzaldehyde **iii**, vanillinezuur **iv**, ficusic zuur **v**, dehydrovomifoliol **vi**, loliolide **vii**, vomifoliol **viii**, boscialin **ix**, 4-hydroxybenzoëzuur **x**, roseoside **xi**, benzyl β-D-glucopyranoside **xii**, protocatechuic zuur **xiii**, kaemferol **xiv**, 2-fenylethyl β-D-glucopyranoside **xv** en 3,3'-di-O-methylellaginezuur 4-O-β-D- xylopyranoside **xvi**. Het moet worden opgemerkt dat al deze verbindingen, behalve 4-hydroxybenzoëzuur **x** en kaemferol **xiv**, voor het eerst werden geïsoleerd uit deze plant, en er geen bewijzen zijn van eerdere gerapporteerde aanwezigheid van megastigmane-type norisoprenoiden in het genus *Gynura*.

Het fytochemische onderzoek van de natuurproducten in *G. divaricata* leidde tot de isolatie van vijfentwintig bestanddelen. Hun structuren werden spectroscopisch bepaald als een organisch zuur (barnsteenzuur **xvii**) en drie van zijn derivaten (ethyl-methyl-succinaat **xix,** methylsuccinaat **xx** en ethylsuccinaat **xxi**), vier fenolzuren (salicylzuur **xviii**, isovanillinezuur **xxii,** 4-hydroxybenzoëzuur **x** en *p*-coumarinezuur **xxiii**), een coumarine (esculetin **xxiv**), vijf flavonoïden (quercetin **xxviii**, kaemferol **xiv**, rutin **xxv**, quercetin 3-O-β-D-glucoside **xxvi** en kaemferol 3-O-β-D-glucoside **xxvii**) en elf kininezuurderivaten (5-O-caffeoylkininezuur **xxxviii**, methyl-5-O-caffeoylquinaat **xxxvi**, 5-O-feruloylkininezuur **xxxvii** en 5-O-*p*-coumaroylkininezuur **xxxv,** 3,4-dicaffeoyl-kininezuur **xxix**, 3,5-dicaffeoylkininezuur **xxx**, 4,5-dicaffeoylkininezuur **xxxi**, methyl-3,4-dicaffeoylquinaat **xxxii**, methyl-3,5-dicaffeoylquinaat **xxxiii**, methyl-4,5-dicaffeoylquinaat **xxxiv** en ethyl-4,5-dicaffeoylquinaat **xxxix**). Betreffende deze componenten, werden verbindingen **x**, **xvii-xxiv**, **xxvi**, **xxix** en **xxxi-xxxix** voor het eerst uit deze plant geïsoleerd.

Alle individuele verbindingen werden getest op de inhibitie van α-glucosidase en Proteïne Tyrosine Fosfatase 1B (PTP1B) betrokken bij de pathogenese van hyperglycemie. Van de geïsoleerde verbindingen, vertoonden 3,4-dicaffeoylkininezuur **xxix**, 4,5-dicaffeoylkininezuur **xxxi**, methyl-3,4-dicaffeoylquinaat **xxxii** en methyl-4,5-dicaffeoylquinaat **xxxiv** significant inhiberende activiteiten tegen α-glucosidase. Bovendien, 3,5-dicaffeoylkininezuur **xxx**, 4,5-dicaffeoylkininezuur **xxxi**, *p*-coumarinezuur **xxiii** en 5-O-*p*-coumaroylkininezuur **xxxv** vertoonden significant inhiberende activiteiten tegen PTP1B. Op basis hiervan werden de hydroxykaneelzuurconjugaten verondersteld

potentieel verantwoordelijk te zijn voor de antidiabetes werkzaamheid van *G. divaricata*. Megastigmane-type norisoprenoïde verbindingen uit *G. bicolor* vertoonden een beperkte activiteit ten opzichte van de twee bovengenoemde enzymen, behalve vomifoliol **viii** dat een sterke inhibitie van PTP1B vertoonde. De biotesting van alle isolaten op meer anti-diabetisch gerelateerde modellen in vitro is interessant voor verder onderzoek.

Chapter 8

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Curriculum vitae

Personal data

Jian Chen
1 Qian Hu Hou Cun, Zhong Shan Men Wai,
Nanjing 210014, China
chenjian9942325@yahoo.com.cn
Jian.Chen@UGent.be

Hangzhou, 02 January 1980
married with Wei-ying Gu
child: Yi-lin Chen (18 October 2009)

Professional experience and education

2010.09-2013.04:	Joint doctoral research, financially supported by the Special Research Fund (BOF-UGent, Belgium)
topic:	Study on the isolation, characterization and possible bioactivity of constituents with hypoglycemic activity from *Gynura divaricata* and *G. bicolor*
promoters:	Prof. Dr. ir. Norbert De Kimpe / Prof. Zheng-tao Wang
locations:	1. Department of Sustainable Organic Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium 2. School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing, China
2008.09-2010.08:	Doctoral graduate program, majoring in Pharmacognosy at China Pharmaceutical University, Nanjing, China
promoter:	Prof. Zheng-tao Wang
2006.07 until now:	Research Associate at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China
2003.09-2006.06:	Master of sciences, majoring in Botany at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China
thesis:	Study on constituents from Loquat Leaf and their hypoglycaemic activity
promoter:	Prof. Wei-lin Li
1999.09-2003.06:	Bachelor of Traditional Chinese Pharmacy
location:	School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing, China

Scientific publications

Publications in international journals with peer review (SCI)

1. **J. Chen**, S. Mangelinckx, A. Adams, W. L. Li, Z. T. Wang, N. De Kimpe. Chemical constituents from the aerial parts of *Gynura bicolor*. *Natural Product Communications*, **2012**, *7*, 1563-1564.
2. **J. Chen**, A. Adams, S. Mangelinckx, B. R. Ren, W. L. Li, Z. T. Wang, N. De Kimpe. Investigation of the volatile constituents of different *Gynura* species from two Chinese origins by SPME/GC-MS. *Natural Product Communications*, **2012**, *7*, 655-657.
3. H. Lü, **J. Chen**, W. L. Li, B. R. Ren, J. L. Wu, H. Q. Zhang. Hypoglycemic effect of the total flavonoid fraction from *Folium Eriobotryae*. *Phytomedicine*, **2009**, *16*, 967-971.
4. H. Lü, **J. Chen**, W. L. Li, B. R. Ren, J. L. Wu, H. Y. Kang, H. Q. Zhang, A. Adams, N. De Kimpe. Hypoglycemic and hypolipidemic effects of the total triterpene acid fraction from *Folium Eriobotryae*. *Journal of Ethnopharmacology*, **2009**, *122*, 486-491.
5. W. L. Li, B. R. Ren, M. Zhuo, Y. Hu, C. G. Lu, J. L. Wu, **J. Chen**, S. Sun. The anti-hyperglycemic effect of plants in genus *Gynura Cass. American Journal of Chinese Medicine*, **2009**, *37*, 961-966.
6. **J. Chen**, W. L. Li, J. L. Wu, B. R. Ren, H. Q. Zhang. Hypoglycemic effects of a sesquiterpene glycoside isolated from leaves of loquat (*Eriobotrya japonica* (Thunb.) Lindl.). *Phytomedicine*, **2008**, *15*, 98-102.
7. **J. Chen**, W. L. Li, J. L. Wu, B. R. Ren, H. Q. Zhang. Euscaphic acid, a new hypoglycemic natural product from *Folium Eriobotryae*. *Pharmazie*, **2008**, *63*, 765-767.

Papers in preparation

1. **J. Chen**, S. Mangelinckx, A. Adams, Z.K. Chen, Z.T. Wang, W.L. Li, N. De Kimpe. Natural flavonoids as potential herbal medication for the treatment of Diabetes Mellitus and its complications.
2. **J. Chen**, S. Mangelinckx, L. Ma, Z.T. Wang, W.L. Li, N. De Kimpe. α-Glucosidase and PTP1B inhibitory caffeoylquinic acids isolated from *Gynura divaricata*.

Abstracts of presentations at conferences

1. **J. Chen**, S. Mangelinckx, A. Adams, W. L. Li, Z. T. Wang, N. De Kimpe. Isolation and identification of the chemical constituents from two *Gynura* species, 17th Symposium on Applied Biological Sciences, Leuven, Belgium, 10/02/2012.
2. **J. Chen**, S. Mangelinckx, A. Adams, W. L. Li, Z. T. Wang, N. De Kimpe. Isolation and characterization of the chemical constituents from *Gynura bicolor* and *G. divaricata*. 8th Joint Meeting of AFERP, ASP, GA, PSE & SIF, New York City, USA, 28/07-01/08/2012.

Participation at conferences and workshops

1. International Forum on Complementary Medicine Research. October 24th-26th, 2008, Nanjing, China.
2. Workshop Medicinal Plants: Tsinghua University - K.U.Leuven collaboration. October 26th, 2010, Leuven, Belgium.
3. The 9th National Symposium on Natural Products Chemistry. November 8th-11th, 2012, Haikou, China.